EVALUATION OF METHANOLIC EXTRACT OF *Hypericum mysorense* OINTMENT FOR ITS WOUND HEALING ACTIVITY

Dissertation submitted to
The Tamilnadu Dr. M.G.R Medical University, Chennai
In partial fulfillment for the requirement of the degree of

MASTER OF PHARMACY

(Pharmaceutics)

MARCH-2014

DEPARTMENT OF PHARMACEUTICS

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPPATTI ROAD, COIMBATORE-641048

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Date:
Place: Coimbatore

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DECLARATION

I do hereby declare that this dissertation entitled "**EVALUATION OF METHANOLIC EXTRACT OF *Hypericum mysorense* OINTMENT FOR ITS WOUND HEALING ACTIVITY** submitted to the TamilNadu Dr.M.G.R.Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutics** was done by me under the guidance of**, Dr.C Sankar M.Pharm., PhD.,** Professor, Department of Pharmaceutics, KMCH College of Pharmacy, Coimbatore, during the year 2013-2014.

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EVALUATION CERTIFICATE

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ABBREVIATIONS USED

MeOH HMS	Methanolic extract of *Hypericum mysorense*
Oint	Ointment
MP	Melting point
e.g.	Example
i.e.	That is
%	Percentage
Kg.	Kilogram
gm.	gram
mg.	Milligram
µg.	Micro gram
ml.	Millilitre
cm.	Centimeter
mm.	Millimeter
nm.	Nanometer
W/W	Weight by weight
W/V	Weight by volume
avg.	Average
hrs.	Hours
pH.	Hydrogen ion concentration
^{o}C	Degree centigrade

RH.	Relative Humidity
RPM.	Revolution per minute
Abs.	Absorbance
Conc.	Concentration
Fig.	Figure
CR	Cumulative Release
R^2	Regression coefficient

ABSTRACT

The majority of the populations in the developing world rely on traditional medicine for their primary healthcare needs. Herbal therapy predominates in traditional medicine as well as in complementary or alternative medicine practiced in the developed world. Among the various indications where traditional herbal medicines are used, skin and skin related disorders are ranked top. Thus, the main objective of the present study is to formulate and evaluate semi-solid dosage forms of *Hypericum mysorense* for its wound healing activity.

The stem of *Hypericum mysorense* was extracted by continuous hot percolation/ soxhletation using methanol as solvent and was evaluated for its phytochemical property, *in-vitro* antimicrobial and *in-vitro* antioxidant activity. Using this methanolic extract, semi-solid dosage form(ointment) was formulated. Ointment was prepared using 2% concentration of the extract by fusion method using emulsifying ointment as base and then tested for physiochemical properties like colour, loss on drying, pH, viscosity, melting point, spreadability and extrudability. HPTLC analysis, *in-vitro* diffusion studies, kinetic studies and stability studies were also conducted on the preparation. The results of *in-vitro* antimicrobial and *in-vitro* antioxidant activity added on to the wound healing activity of the extract. Hence, the ointment containing 2% methanolic extract of *Hypericum mysorense* was evaluated for its wound healing activity using excision wound model. The formulation did not produce any skin irritation for about a week when applied over the skin. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug, povidone iodine ointment. Therefore, the present study concluded that the *Hypericum mysorense* at 2% concentration of the methanolic extract formulated as an ointment showed a better wound healing formulation for better patient care and pharmaco economical.

Keywords: *Hypericum mysorense*, Excision wound model, ointment, *in-vitro* antimicrobial, *in-vitro* antioxidant

INTRODUCTION

OVERVIEW OF TRADITIONAL HERBAL MEDICINE[1]:

India is a birthplace of indigenous medicine such as Siddha, Ayurveda, and Unani where many herbs have been used for treatment of human ailments. About 65% of total global population remains dependent on traditional medicines for their primary healthcare. Herbs are occupying a comeback and an 'Herbal Renaissance' is blooming across the world. They have been evidently prized for their medicinal, flavoring and aromatic qualities for centuries, yet for a while they were over shadow by synthetic products of modern civilization. Folk medicine is generally defined as traditional medicine that is practiced by non-professional healers or embodied in local custom or lore, generally involving the use of natural and especially herbal remedies. The World Health Organization (WHO) defines traditional medicine as "the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercise, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being." Treatments are specific to a particular ailment and may include the power of rare, dancing, sweet baths, massage, medicinal herbs, hot and cold foods or other means not practiced in modern medicine.

Once having realized their sources and adverse effects, people are going back to nature with hopes of safety and security. The rich treasure of herbal drugs is forming a boon for our society. Plant derived compounds, apart from their nutritive values, could serve as important therapeutic weapons to fight various human and animal diseases, thereby making them indispensable in traditional medicine for treating a number of diseases. Plant drugs, popularly known as herbal medicines have since been unabatedly used to treat various diseases. The major challenge is to protect traditional knowledge and will prove to be a beneficial asset to our human surrounding. For all the ailments herbal formulations are proved to be effective without any side effects commonly seen with allopathic drugs.

OINTMENT[2]

An ointment is a homogeneous, viscous, semi-solid preparation, most commonly greasy, thick oil (oil 80% - water 20%) with a high viscosity which is intended for external application to the skin or mucous membranes. Ointments have a water number that defines the maximum amount of water that it can contain. They are used as emollients or for the application of active ingredients to the skin for protective, therapeutic, or prophylactic purposes and where a degree of occlusion is desired.

Ointments are used topically on a variety of body surfaces. These include the skin and the mucous membranes of the eye (an eye ointment), vagina ,anus, and nose. An ointment may or may not be medicated.

Ointments are usually very moisturizing, and good for dry skin. They have a low risk of sensitization due to having few ingredients beyond the base oil or fat, and low irritation risk. There is typically little variability between brands of generics and name brand drugs. They are often disliked by patients due to greasiness.

OINTMENT BASE[3]:

The vehicle of an ointment is known as the ointment base. The choice of a base depends upon the clinical indication for the ointment. There are five classes or types of ointment bases which are differentiated on the basis of their physical composition. These are:

- oleaginous bases
- absorption bases
- water in oil emulsion bases
- oil in water emulsion bases
- water soluble or water miscible bases

Each ointment base type has different physical characteristics and therapeutic uses based upon the nature of its components which is summarized in the table below.

SUMMARY CHART: PROPERTIES OF OINTMENT BASES

	Oleaginous ointment bases	**Absorption ointment bases**	**Water/Oil Emulsion ointment bases**	**Oil/Water Emulsion ointment bases**	**Water-miscible ointment bases**
Composition	oleaginous compounds	oleaginous base + w/o surfactant	oleaginous base + water (< 45% w/w) + w/o surfactant (HLB ≤8)	oleaginous base + water (> 45% w/w) + o/w surfactant (HLB ≥9)	Polyethylene Glycols (PEGs)
Water Content	Anhydrous	Anhydrous	Hydrous	Hydrous	anhydrous, hydrous
Affinity for Water	Hydrophobic	Hydrophilic	Hydrophilic	Hydrophilic	Hydrophilic
Spreadability	Difficult	Difficult	Moderate to easy	Easy	Moderate to easy
Washability	Nonwashable	Nonwashable	non- or poorly washable	Washable	Washable
Stability	oils poor; hydrocarbons better	oils poor; hydrocarbons better	unstable, especially alkali soaps and natural colloids	unstable, especially alkali soaps and natural colloids; nonionic better	Stable
Drug Incorporation Potential	solids or oils (oil soluble only)	solids, oils, and aqueous solutions (small amounts)	solids, oils, and aqueous solutions (small amounts)	solid and aqueous solutions (small amounts)	solid and aqueous solutions
Drug Release Potential*	Poor	poor, but > oleaginous	fair to good	fair to good	Good
Occlusiveness	Yes	Yes	sometimes	No	No

Uses	protectants, emollients (+/-), vehicles for hydrolysable drugs	protectants, emollients (+/-), vehicles for aqueous solutions, solids, and non-hydrolysable drugs	emollients, cleansing creams, vehicles for solid, liquid, or non-hydrolysable drugs	emollients, vehicles for solid, liquid, or non-hydrolysable drugs	drug vehicles
Examples	White Petrolatum, White Ointment	Hydrophilic Petrolatum, Anhydrous Lanolin, Aquabase™, Aquaphor®, Polysorb®	Cold Cream type, Hydrous Lanolin, Rose Water Ointment, Hydrocream™, Eucerin®, Nivea®	Hydrophilic Ointment, Dermabase™, Velvachol®, Unibase®	PEG Ointment, Polybase™

*Varies depending upon specific content of the ointment base and the relative polarity of the drug(s) incorporated. This table refers more generally to the release of a typical nonelectrolyte (primarily lipophilic) drug.

METHODS OF PREPARATION OF OINTMENT:

- Trituration: In this method, finely subdivided insoluble medicaments are evenly distributed by grinding with a small amount of the base followed by dilution with gradually increasing amounts of the base.
- Fusion: In this method, the ingredients are melted together in descending order of their melting points and stirred to ensure homogeneity.

EVALUATION OF OINTMENT:

1. Colour and odour
2. Loss on drying
3. pH
4. Spreadability
5. Extrudability

6. Diffusion Study
7. Stability Study

IN-VITRO DIFFUSION STUDY:

The *in-vitro* permeation study was carried out by using excised rat abdominal skin and franz diffusion cell. The skin was sandwiched between donor and receptor compartments of the diffusion cell. A 2.2 cm diameter patch was placed in intimate contact with the stratum corneum side of the skin; the top side was covered with aluminium foil as a backing membrane. Teflon bead was placed in the receptor compartment filled with 12ml of normal saline. The cell contents were stirred with a magnetic stirrer and a temperature of 37 ± 5°C was maintained throughout the experiment. Samples of 1ml were withdrawn through the sampling port at different time intervals for a period of 24 hours, simultaneously replacing equal volume by phosphate buffer pH 7.4 after each withdrawal. Then the samples were analyzed by spectrophotometrically.

OINTMENT APPLICATIONS:

There are various parts of the body surfaces, skin and mucous membranes where ointment is applied for curing certain skin or disease conditions. Ointment is applied on hands, legs, face, eyes, ears, vagina, anus, throat etc. There are various problems when an ointment is suggested for treatment such as

- Ointment for burns
- Ointments for cuts
- Ointments for pain
- Ointments for itching
- Ointments for inflammation and pain
- Ointments for boils and scars
- Ointments for skin problems like eczema, dermatitis and psoriasis

CONTAINERS:

Ointments are packaged either in ointment jars or tubes. The ointment jars are usually straight-sided screw cap jars of glass or plastic. The ointment should be packed while it is still warm and fluid by either pouring it directly into the jar or packaging it by pressure with a spatula. Ointment jars hand-filled by the pharmacist should also be finished to avoid contact between the ointment and the cap. This can be accomplished by forcing a spatula across the ointment jar while depressed slightly in the ointment.

Ordinary tin tubes are used for packaging ointments if the ointment is not too stiff. These tubes are available in various sizes complete with ophthalmic, rectal, vaginal, or plain tips. Tubes are usually filled at the prescription counter by rolling the ointment into a cylinder in a piece of parchment paper in such a way that it can be inserted into the tube. The paper can be removed by carefully flattening the open end of the tube against the paper and withdrawing it while firmly holding the end of the tube together. It is usually necessary to remove the cap of the tube while filling to avoid air pockets in the tube.

ANTIMICROBIAL THERAPY[4]

There is a growing awareness of active contribution of the environment of context of infection. The nature of micro organism and many occasions on which they come into contact with humans, while others go on to produce diseases, lies in the nature of interaction between the microbe and host. The basis of antimicrobial therapy is to arrest the growth of the infecting micro organism and where possible to kill it without damage to patient. Once the decision to treat infection has been taken, the formulation has been formulated it is necessary to predict the micro organism. Antimicrobial agent's particularly antibacterial agents can be effective against a wide range of micro organism.

Bacterial skin infections can be distinguished by clinical examination and simple laboratory investigations. These are very common; they range from annoying to deadly. Most bacterial infections of the skin are caused by *Staphylococcal aureus, Streptococcus* and *Pseudomonas aeruginosa. Staphylococcal aureus* bacteria are classified as gram positive cocci based on their appearance under a microscope. They may occur singly or

grouped in pairs, short chains or grape-like clusters. They are only able to invade via broken skin or mucous membranes; hence intact skin is an excellent human defence. Once they have invaded they have various ways to avoid host defense. They :

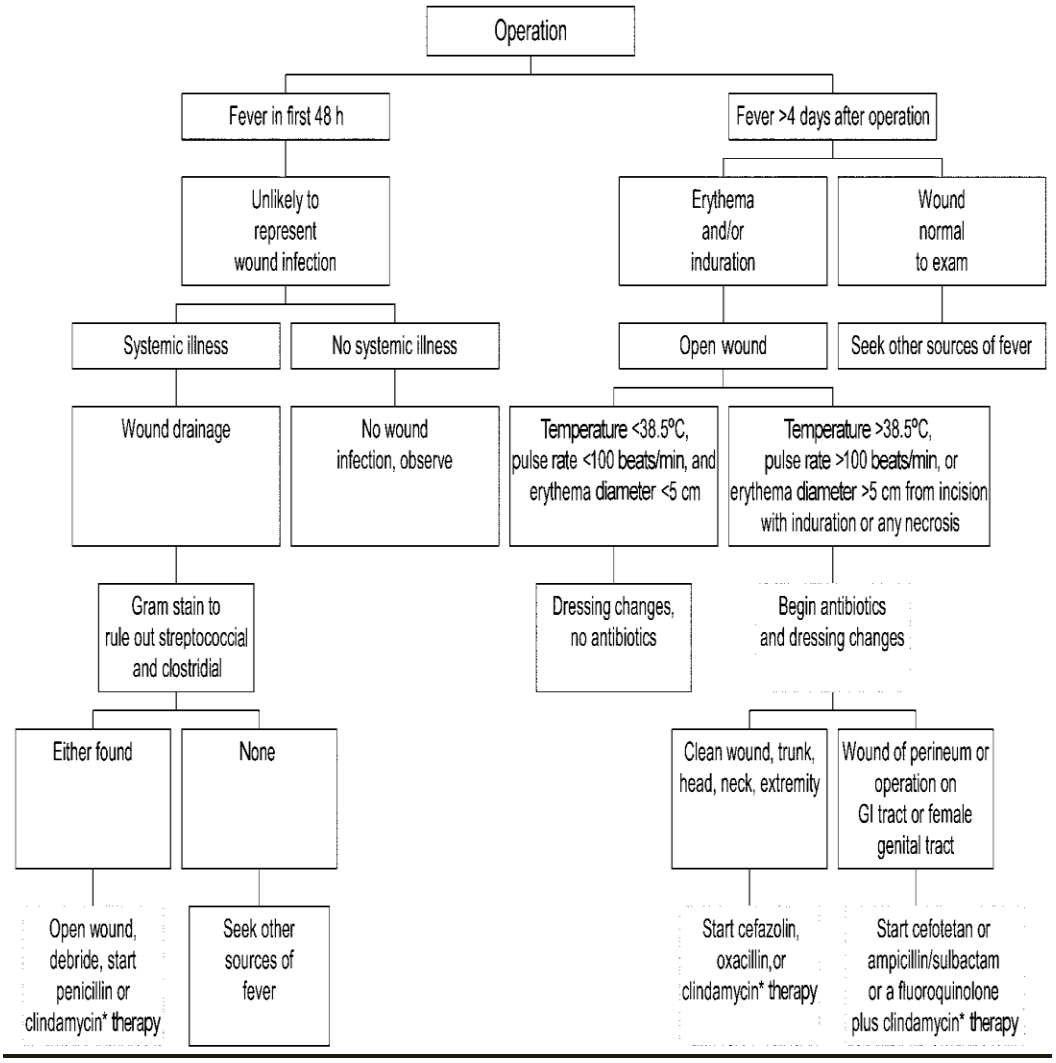
- Hide their antigen to avoid an immune response
- Kill infection- fighting cells (phagocytes)
- Survive within host infection- fighting cells
- Develop resistance to antibiotics.

MANAGEMENT OF BACTERIAL SKIN INFECTIONS:

- Appropriate antibiotics
- Drainage of pus collections
- Surgical removal (debridement) of dead tissue (necrosis)
- Removal of foreign bodies (eg. Stitches) that may be a focus of persisting infection

Due to widespread antibiotic resistance, it is better to prevent Staphylococcal infections wherever possible. The most effective way is to wash hands before touching broken skin. Various antibiotics are used depending upon site and severity of infection and susceptibility testing. It is also important to clear bacteria colonizing the nostrils and under the finger nails with either antibiotic ointment (e.g. fusidic acid or mupirocin) or petroleum jelly several times daily for one week of each month. The algorithm presented provides an approach to diagnosis and treatment.

Figure1.Algorithm showing approach to diagnosis and treatment

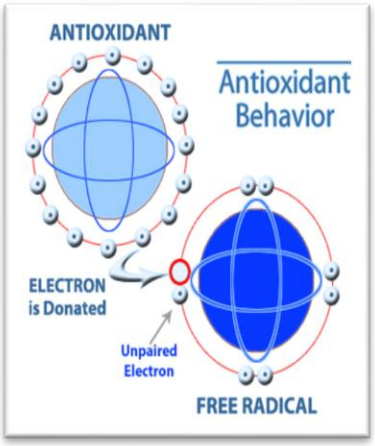


ANTIOXIDANT ACTIVITY[5]:

An antioxidant is a molecule capable of inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reaction can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols.

Reactive oxygen species (ROS), sometimes called active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions (O_2) and hydroxyl radicals (OH) as well as non free-radical species such as hydrogen peroxide(H_2O_2). In living organisms various ROS can form in different ways, including normal aerobic respiration stimulated polymorphonuclear leukocytes and macrophages, and peroxisomes. These appear to be the main endogenous source of most of the oxidants produced by cells. Exogenous sources of free radical include tobacco smoke, ionizing radiation, certain pollutants, organic solvents and pesticides. Free radicals can cause lipid peroxidation in foods, which leads to their deterioration. In addition, reactive oxygen species have been implicated in more than 100 diseases, including malaria, AIDS, heart diseases, stroke, atherosclerosis, diabetes and cancer. When produced in excess, ROS can cause tissue injury). In a normal cell, there are appropriate oxidants: antioxidant balance. However, this balance can be shifted, when production species is increased or when levels of antioxidants are diminished. This stage is called oxidative stress. Oxidative stress results in the damage of biopolymers including nucleic acid, proteins, polyunsaturated fatty acid and carbohydrates. Lipid per oxidation is oxidative detoriation of polyunsaturated lipids and it involves ROS and transition metal ions. It is a molecular mechanism of cell injury leading yield a wide range of cytotoxic products, most of which are aldehyde, like malondialdehyde. Oxidative stress causes serious cell damage leading to variety of human diseases like Alzheimer's disease, Parkinson's disease, Arthritis and neuron de generative disorder etc. However, tissue injury can itself cause ROS generation. Nevertheless, all aerobic organisms, including human beings, have antioxidant defences that protect against oxidative damages, and numerous damage removal and repair enzymes to remove or repair damaged molecules. However, this natural antioxidant mechanism can be inefficient, and hence dietary intake of antioxidant compounds is important. There are some synthetic antioxidant compounds such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole(BHA), commonly used in processed foods.

Figure 2: Mechanism of Antioxidant



IN-VITRO DETERMINATION OF ANTI OXIDANT CAPACITY

This approach has benefits over simply quantifying antioxidant components as it provides a measure of their effectiveness. The various conventional and latest methods come under *in-vitro* methods. It is very difficult to select a suitable anti oxidant assay method. Antioxidant act by several mechanisms and no one assay can capture the different modes of action of antioxidant. Conventional cuvette assay of radical scavenging activity is replaced by 96-well plate titer assay from past couple of years. Cuvette assay method uses UV-visible spectrophotometer to see the absorbance, where as 96-well plate method uses ELISA plate reader for absorbance.

WOUND HEALING ACTIVITY[6]:

Wounds are inescapable events in life. Wounds may arise due to physical, chemical or microbial agents. Wound healing involves a complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix. The phases of normal wound healing include hemostasis, inflammation, proliferation and remodeling. Each phase of wound healing is distinct, although the wound healing process is continous, with each phase overlapping the next. Because successful wound healing requires adequate blood and nutrients to be supplied to the site of damaged tissue.

CLASSIFICATION OF WOUNDS:

Wounds are classified as open wounds and closed wounds on the basis of underlying cause of wound creation and as acute and chronic wounds on the basis of physiology of wound healing

(a) Open Wound[7]:

Though an open wound blood escapes the body and bleeding is clearly visible. Open wound is further classified as:

- Incised Wound:

  It is an injury with no tissue loss and minimal tissue damage. It is caused by a sharp object such as knife. Bleeding in such cases can be profuse, so immediate action should be taken.

- Abrasions or Superficial Wounds:

  It is caused by sliding fall onto a rough surface. During abrasion the topmost layer of the skin i.e. epidermis is scraped off that exposes nerve ending resulting in a painful injury. Blood loss similar to a burn can result from serious abrasions.

- Laceration Wound or Tears Wounds:

  This is the nonsurgical injury in conjunction with some type of trauma, resulting in tissue injury and damage.

- Puncture Wounds:

  They are caused by some object puncturing the skin, such as needle or nail. Chances of injection in them are common because dirt can enter into the depth of wound.

- Gunshot Wounds:

  They are caused by a bullet or similar driving into or through the body.

- Penetration Wounds:

  Penetration wounds are caused by an object such as a knife entering and coming out from the skin.

(b) Closed Wound:

In closed wounds blood escapes the circulating system but remain in the body. It includes contusion or bruises, heamatomas or blood tumor, Crush injury etc.

- Contusions or bruises:

  Bruises are caused by a blunt force trauma that damage tissue under the skin.

- Hematomas or blood tumor:

  They are caused by damage to a blood vessel that consequently causes blood to collect under the skin.

- Crush injury:

  Crush injury is caused when great or extreme amount of force is applied on the skin over long period of time.

- Acute Wounds:

  Acute wound is a tissue injury that normally proceeds through an orderly and timely reparative process that results in sustained restoration of anatomic and functional integrity. Acute wounds are usually caused by cuts or surgical incisions and complete the wound healing process within the expected time frame.

- Chronic Wounds:

  Chronic wounds are wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation chronic wounds either require a prolonged time to heal or recur frequently. Local infection, hypoxia, trauma, foreign bodies and systemic problems such as

diabetes mellitus, Malnutrition, immunodeficiency or medications are the most frequent causes of chronic wound.

FACTOR AFFECTING WOUND HEALING[8]:

- Improper diet
- Infection at the wound site
- Insufficient oxygen supply and tissue perfusion to the wound area
- Drugs
- Elderly age
- Diabetes and other diseases conditions

Wound healing is normal biological process in the human body. Many factors can adversely affect this process and lead to improper and impaired wound healing.

Improper Diet:

Wound healing is anabolic process that requires both energy and nutritive substrates. It is reported that serum albumin level of 3.5gm/dl or more is necessary for proper wound healing. Protein is essential for collagen synthesis on wound site. A state malnutrition may provide an inadequate amount of protein and this can decreased the rate of collagen synthesis wound tensile strength or increased chance of infection

Infection at the Wound Site:

Wound infection is probably the most common reason of impaired wound healing. *Streptococcus aeureus, Streptococcus pyrogens, Escherichia coli* and *Pseudomonas aeruginosa.*

Insufficient Oxygen Supply and Tissue Perfusion to the Wound Area:

Adequate blood supply and tissue perfusion is extremely important for wound healing. Excessive pain, cold and anxiety can cause local vasoconstriction and increased healing time. Smoking and use of tobacco decreased tissue perfusion and oxygen tension in wound.

Drugs:

Many drugs are known to impair wound healing. Chemotherapeutic drugs are used in cancer are the largest group well known to delay wound repair. Systemic glucocorticoids interfere normal healing process by reducing collagen synthesis and fibroblast proliferation.

Elderly Age:

Elderly age is found to be associated with delay wound healing. It is reported that the fibroblast growth and activity diminishes and collagen production, wound contraction is slow in older individuals

Diabetes and Other Diseases:

Diabetic patients are more susceptible to wound healing. In study wound infection rate was found 11% higher in diabetic patients than in general patient's population. Acute and chronic liver diseases also associated with delay wound healing. Patients with altered immune faction have an increased susceptibility to wound infection.

PHASES INVOLVED IN WOUND HEALING:

Wounding

↓ Calendulus succus – topical antimicrobial

Hemostasis

↓

Inflammatory Phase

↓ Vitamin A- enhances early inflammatory phase

Brometalin – prevent prolonging inflammatory phase

Vitamin C –enhances neutrophil migration

Proliferative phase

↓ VitaminC-necessary for collagen synthesis

Centilla asiatica-promotes type- 1 collagen synthesis

Glucosamine – enhances hyaluronic acid production

VitaminA – promotes epithelial cell differentiation.

Zinc – required for DNA synthesis,cell division.

Calendula succus and aloevera: support granulation

Remodelling (Protein deficiency) – inhibits wound remodeling

THE INFLAMMATORY PHASE[9]:

The inflammatory phase starts immediately after the injury that usually last between 24 and 48 hrs and may persist for up to 2 weeks in some cases the inflammatory phase launches the haemostatic mechanisms to immediately stop blood loss from the wound site. Clinically recognizable cardinal sign of inflammation, rubor, calor, tumor, dolor and function-laesa appear as the consequence. This phase is characterized by vasoconstriction and platelet aggregation to induce blood clotting and subsequently vasodilatation and phagocytosis to produce inflammation at the wound site.

FIBROBLASTIC PHASE[10]:

The second phase of wound healing is the fibroblastic phase that lasts upto 2 days to 3 weeks after the inflammatory phase. This phase comprises of three steps viz. granulation, contraction and epithelialisation. In the granulation step fibroblasts form a bed of collagen and new capillaries are produced. Fibroblast produces a variety of substances essential for wound repair including glycosaminoglycans and collagen. Under the step of contraction wound edges pull together to reduces the defects in the third step epithelial tissues are formed over the wound site.

EPITHELIZATION PHASE[11]:

Epithelial cell migration is one of the vital processes of wound healing. The stem cells of epithelium must detach from the edges of the wound and migrate into wound. Normally dermal basal cells adhere to each other and to the underline basal layer of the dermis. Following mobilization, epithelial cells begin to enlarge and migrate down and across the wound. Transected hair follicles also contribute to the number of migrating epithelial cells. Epithelial cell migrating across wound usually move along the basal lamina or fibrin deposits, this phenomenon is called contact guidance and is an important factor in epithelial migration. Epithelial migration is followed by increased mytosis of epithelium. Recent evidence suggests that a water soluble heat labile substance called chalcone which is secreted at the wound site is responsible for regulation for mytosis.

PROLIFERATIVE PHASE[12]:

Proliferative Phase (2 days to 3 weeks) includes:

- Granulation stage: Fibroblasts lay bed of collagen fills defect and produces new
- Contraction stage: Wound edges pull together to reduce defect
- Epithelialization stage: Crosses moist surface cell travel about 3 cm from point of origin in all directions

CONTRACTION PHASE[13]:

Wound contraction is caused by the action of differentiated fibroblasts (myofibroblasts) in the granulation tissue, which contain filaments of smooth muscle actin. Contraction of these fibroblasts makes the wound margins move toward the center of the wound. Wound contraction started sooner in ponies than in horses and it was significantly more pronounced in ponies. Additionally, it was significantly more pronounced in body wounds compared with the limb wounds. As a result, second intention wound healing was significantly faster in ponies than in horses, and significantly faster in body wounds than in metatarsal wounds. Histology showed that myofi-broblasts were more organized in the wounds of the ponies: the myofibroblasts in the newly formed granulation tissue were transformed into a regularly organized pattern within 2 weeks, in which the cells were orientated perpendicular to the vessels and parallel to the wound surface. This appears to be a more favorable condition for wound contraction to occur. In the horses, myofibroblast organization took much longer. No differences were found in the number of fibroblasts, the amounts of smooth muscle actin and collagen. Further research was performed to investigate whether the differences in wound contraction between horses and ponies were caused by differences in the inherent contraction capacity of fibroblasts or the local environment of the fibroblasts. It was found that no differences existed in the inherent contraction capacity of fibroblasts from ponies and horses in vitro. However, the level of Transforming Growth Factor,the most important instigator of wound contraction, was significantly higher in the granulation tissue of pony wounds compared with horse wounds.

REMODELING PHASE[14]:

This phase last for 3 weeks to 2 years. New collagen is formed in this phase. Tissue tensile strength is increased due to intermolecular cross-linking of collagen via vitamin-C dependent hydroxylation. The scar flattens and scar tissues become 80% as strong as the original.

The wound healing activities of plants have since been explored in folklore. Many ayurvedic herbal plants have a very important role in the process of wound healing.

Plants are more potent healers because they promote the repair mechanisms in the natural way. Extensive research has been carried out in the area of wound healing management through medicinal plants. Herbal medicines in wound management involve disinfection, debridement and providing a moist environment to encourage the establishment of the suitable environment for natural healing process.

ACTIVITIES LEADING TO WOUND HEALING:

(a) Anti-inflammatory Activity:

The acute inflammatory response during the early stages of injury generates factors that are essential for tissue growth and repair. When prolonged, however, chronic inflammation can be detrimental, preventing wound remodeling and matrix synthesis, leading to delay in wound closure and an increase in wound pain. Thus, it is possible that an anti-inflammatory effect could facilitate wound healing and improve patient comfort, although traditional texts and animal studies indicate that extracts exert an anti-inflammatory effect.

(b) Antioxidant Effect:

The production of free radicals at or around the wound bed may contribute to delay in wound healing through the destruction of lipids, proteins, collagen, proteoglycan and hyaluronic acid. Agents that demonstrate a significant antioxidant activity may, therefore, preserve viable tissue and facilitate wound healing.

(c) Antimicrobial Activity:

Wound healing can also be delayed when micro organisms are present in large enough numbers. Therefore, reducing the bacterial load of a wound may be necessary to facilitate wound healing as well as to reduce local inflammation and tissue destruction. An ideal agent for the prevention and control of wound infection would therefore be one that directly destroys the pathogens while also stimulate immune activity.

(d) Analgesic Activity:

The open wounds can generate pain and subsequent disability, it is important that the dressing applied does not increase pain, and, if possible, it should lessen the pain.

REVIEW OF LITERATURE

Pulok K .Mukherjee *et al* [15] studied the *in-vivo* wound healing activity of leaf extract of *Hypericum mysorense* with different wound model in rats. The methanol extract of leaves of Hypericum mysorense, in the form of ointment in two different concentrations (5% and 10% w/w ointment of aerial part extract in simple ointment base) was evaluated for wound healing potential in excision wound model and incision wound model in rats. Both the concentrations of the methanol extract ointment showed significant responses in both the wound types tested when compared with the control group. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time, regeneration of tissues at wound site, tensile strength of the wound and histopathological characteristics were comparable to those of a standard drug Nitrofurazone ointment.

Shilpashree H.P *et al* [16]studied the *in vitro* plant regeneration and accumulation of flavonoids in *Hypericum mysorense,* which could be used for large scale production in pharmaceuticals.

A.A. Leslie Gunatilaka, *et al*[17] isolated and characterized simple xanthones from the phenolic fraction of the chloroform extract of the timber of *Hypericum mysorense*. The presence of simple xanthones in this genus supports the classification of *Hypericum* in the subfamily Hypericoideae in Guttiferae.

Shanmugam Moorthi *et al*[18] studied the effect of ethanolic extract of the *Hypericum mysorense* on antioxidant and antidepressant activity in the liver and brain of reserpine induced rats. Oral administration of ethanolic extract of Hypericum mysorense (0.12g/kg) for 30 days to reserpine induced rats caused a significant increase in superoxide dismutase, catalase, glutathione peroxidase, glutathione – S – transferase, glutathione reductase, glucose 6 phosphate dehydro-genase, glutathione, vitamin C, vitamin E, total sulfhydryl group, non protein sulfhydryl group in liver and brain of reserpine induced rats. The results indicate the antioxidant and antidepressant effect of ethanolic extract of Hypericum mysorense.

P. Vijayan, C. Raghu *et al*[19]screened various plant extracts for antiviral properties by cytopathic effect inhibition assay and virus yield reduction assay and determined that *Hypericum mysorense* exhibited significant antiviral activity at a concentration non toxic to the cell line used.

Atul Murlidhar Wahile [20]investigated the analgesic potential of ethyl acetate and methanolic extracts of *Hypericum mysorense* through hot plate method and acetic acid induced writhing test in mice.

H. Raghu Chandrasekhar *et al*[21] carried out a study on methanolic extracts of different parts of *Hyperium mysorense* namely aerial parts, flowering tops, leaf, root and stem for its *in vitro* and *in vivo* anti cancer activities.

Atul Murlidhar Wahile[20] , proved that in carrageenan-induced rat paw edema model, ethyl acetate extract of *Hypericum mysorense* showed significant ($P<0.05$) anti-inflammatory activity by inhibiting inflammation.

S. Gopinath *et al*[22] carried out to determine the possible bioactive components of leaf and bark of *Hypericum mysorense* using GC-MS.

A. K Singhal *et al* [23]evaluated the wound healing property of ethanolic extract of *Argyreia nervosa* in normal and diabetic animals by oral and topical administration and demonstrated that *A. nervosa* leaves extract applied topically promotes healing of wounds more significantly as compared to oral application, in both normal rats and alloxan induced diabetic rats, where healing is otherwise delayed

A. Jaswanth *et al* [24]studied the wound healing effect of methanolic extract of the root of *Aegle marmelos* by formulating it in the form of an ointment with two different concentrations (5% and 10% w/w in simple ointment base) in excision wound model and incision wound model in rats. In both the concentrations, the extract ointment produced a significant response in both the wound types tested, as evidenced by its wound contracting ability, wound closure time and increase in the tensile strength. The results were also comparable to those of a standard drug nitrofurazone.

Mohammad Zuber *et al*[25] isolated the bulbs of *Allium sativum*(Family:Liliaceae) and screened for wound-healing activity on the Swiss albino rats by excision wound model and incision wound model respectively. The studies on excision wound model reveals significant wound healing activity of the extract, which is comparable with the reference control framycetin.

Ahmad Oryan *et al*[26] evaluated the wound healing properties of *Aloe vera* (Aloe barbadensis) on cutaneous wounds. The lesions of the treated animals showed a better alignment, fewer inflammatory cells infiltration and significantly improved biomechanical properties on day 20 ($P<0.05$). These results suggested that application of *Aloe vera* aqueous extract on open wounds induces significant wound contraction and accelerates healing.

C C Barua *et al*[27], worked on wound healing activity of methanolic extract of leaves of *Alternanthera brasiliana* Kuntz using *in vivo* and *in vitro* models and proved to have significant effect.

Nayak B S *et al* [28] ,study aimed to evaluate the wound-healing activity of fruit extract of *Persea americana* (avocado) in rats .The extract-treated wounds were found to epithelialise faster than the controls ($p < 0.001$).

C C Barua *et al*[29], study was aimed to evaluate the wound healing activity of the methanol extracts of leaves of *Azadirachta indica (neem) and Tinospora cordifolia (guduchi)* using excision and incision wound models in Sprague Dawley rats. The study thus revealed promising wound healing activity of methanolic extract of *A. indica and T. cordifolia* and provides a scientific rationale for the traditional use of these plants in the management of wounds.

A Osunwoke Emeka *et al*[30], investigated The wound healing effect of the aqueous leaves extracts of *Azadirachta indica* on adult male wistar rats. The extracts of *Azadirachta indica* significantly increased ($p<0.05$) the day of complete wound closure in experimental group compared to control group. Aqueous leave extracts of *Azadirachta*

indica promotes wound healing activity through increased inflammatory response and neovascularization.

S.K. Purohit *et al*[31], undertook an evaluation on *Azadirachta indica* (Neem) leaves ethanolic extract for wound healing activity through topical route on excision wound model. The activity was compared with standard drug Povidone Iodine ointment (5% w/w). *Azadirachta indica* leaves ethanolic extract was found to have better and faster wound healing effect than standard drug Povidone Iodine ointment on excision wound model.

S. Murthy *et al* [32], carried out evaluation of *in vivo* wound healing activity of *Bacopa monniera* on different wound model in rats. It decreased myeloperoxidase and free radical generated tissue damage, promoting antioxidant status, faster collagen deposition, other connective tissue constituent formation, and antibacterial activity.

M. Saroja *et al*[33], carried out a study on the wound healing activity of flavonoid fraction of *Cynodon dactylon* in Swiss albino rat. The study revealed that the wound healing activity may be due to free radical scavenging activity of flavonoid fraction.

Shivananda Nayak *et al*[34], evaluated the wound healing activity of *Allamanda cathartica. L.* and *Laurus nobilis. L.* extracts on rats. The data of this study indicated that the leaf extract of *Allamanda* possesses better wound healing activity than the *Laurus nobilis* and it can be used to treat different types of wounds in human beings too.

Renu Solanki *et al*[35], carried out a study on ethanolic extract of leaves of *Aegle marmelos* and *Ocimum basilicum* for its wound healing activity through topical route on excision wound model. The activity was compared with standard drug povidone iodine ointment (5% w/w) respectively. The leaves of*Aegle marmelos* ethanolic extract was found to have better wound healing effect than ethanolic extract of leaves of *Ocimum basilicum* on excision wound model. The leaves of *Aegle marmelos* ethanolic extract also showed faster rate of healing when compared with standard topical application.

Vipin Kumar Garg *et al*[36], The wound-healing efficacy of ethanolic and aqueous extracts of *Ficus benghalensis* was evaluated in excision and incision wound models. The parameters studied include rate of wound contraction, period of complete epithelialization, and tensile strength of incision wound. Student's *t* test was used to analyze the results obtained from the present study and $P<0.05$ was considered significant. Both the ethanolic and aqueous extracts of *F. benghalensis* were found to possess significant wound-healing activity.

B Shivananda Nayak *et al*[37] , worked on the aqueous extract of *Carica papaya* fruit for its wound healing activity in streptozotocin- induced diabetic rats using excision and dead space wound model. The extract treated wounds epithelize faster than control.

S Ambiga *et al*[38], isolated the flowers of *Ipomoea carnea*(Family: Convolvulaceae) , and was screened for wound-healing activity on the male wistar rats by excision wound model and incision wound model respectively. The studies on excision wound model reveals significant wound healing activity of the extract, which is comparable with the reference control sulphathiazole.

Kokane DD *et al* [39] , studied on the roots of *Mimosa pudica* for wound healing activity by incorporating the methanolic and the total aqueous extract in simple ointment base B.P in concentration of 0.5% (w/w), 1% (w/w) and 2% (w/w) using three types of model in rats viz. excision, incision and estimation of biochemical parameter. The methanolic extract exhibited good wound healing activity probably due to phenols constituents.

Mahmood A. Abdulla *et al* [40], assessed the effects of topical application of *Rafflesia hasseltii* buds and flowers extract on the rate of wound healing and histology of healed wound , the findings of increased rate of wound closure and contraction together with the histological findings suggest that *Rafflesia hasseltii* buds extract is very effective in accelerating the wound healing process.

Kotade Kiran *et al* [41], studied on the wound healing activity of *Sesamum indicum* L seed and oil in rats using *Aloe vera* as standard healing agent and the result suggested excellent wound healing property.

Agren M S *et al* [42], investigated the effect of topically applied zinc on leg ulcer healing and examined its effect on some mechanisms in wound healing using standardized animal models.

K N Chidambara Murthy *et al* [43]**,**carried out a study on the methanolic extract of dried pomegranate (*Punica granatum*) peels by formulating it as a 10% (W/W) water-soluble gel for its wound healing property against an excision wound on the skin of Wistar rats. The activity was compared with that of a commercial topical antibacterial applicant. The wound healing activity was assessed by measuring the percent contraction in skin and estimation of collagen content in terms of hydroxyproline content.

AIM AND OBJECTIVE

Aim:

The present studies aim is to highlight the use of *Hypericum mysorense* for the treatment of cuts and wounds as a wound healer. The study involves the formulation and evaluation of methanolic extract of *Hypericum mysorense* as a semi-solid dosage form for its wound healing activity.

Objective:

- To carry out continous hot percolation of *Hypericum mysorense* using methanol as solvent.
- To evaluate phytochemical constituents of the obtained methanolic extract of *Hypericum mysorense.*
- To evaluate the methanolic extract of *Hypericum mysorense* for its *in-vitro* antioxidant and antimicrobial property.
- To formulate methanolic extract of *Hypericum mysorense* as a semi-solid dosage form (ointment).
- To evaluate the physiochemical parameters of the formulated ointment.
- To conduct HPTLC analysis, *in-vitro* diffusion studies, release kinetics and stability studies of the prepared ointment.
- To assess the wound healing property of the prepared herbal ointment by excision wound model.

PLAN OF WORK

1. Extraction of *Hypericum mysorense* by continous hot percolation using methanol as solvent.
2. Phytochemical analysis of the methanolic plant extract.
3. Assessment of the *in-vitro* antimicrobial screening by disc diffusion method.
    a) Anti- bacterial Screening
    b) Anti-fungal Screening
4. Evaluation of *in-vitro* antioxidant property of the methanolic extract.
    a) DPPH Assay
    b) FRAP Assay
5. Formulation of methanolic extract of *Hypericum mysorense* as a semi-solid dosage form (ointment).
6. Evaluation of the physiochemical parameters of the formulated ointment.
    a) Colour and odour
    b) Loss on drying
    c) pH
    d) Spreadability
    e) Extrudability
    f) Stability Study
7. Carry out the HPTLC analysis diffusion studies release kinetics and stability studies of the prepared semi-solid dosage form.
8. Assessment of wound healing property of the prepared herbal ointment by excision wound model.

PLANT PROFILE[44,45]

COLLECTION AND AUTHENTICATION:

The plant *Hypericum mysorense* was collected in and around Ooty. The taxonomical identification of plant was authenticated by Dr. S. Rajeswari, Department of Botany, PSG College of Arts and Science, Coimbatore.

DESCRIPTION OF PLANT'S BOTANICAL INFORMATION:

Botanical name : *Hypericum mysorens*e
Family : Hypericaceae

CLASSIFICATION:

Kingdom	: Plantae
Sub Kingdom	: Tracheobionta
Super division	: Spermatophyta
Division	: Magnoliophyta
Class	: Magnoliopsida
Subclass	: Dilleniidae
Order	: Malpighiales
Family	: Hypericaceae

Subfamily	: Hypericoideae
Tribe	: Hypericeae
Genus	: *Hypericum*
Species	: *mysorense*
Habit	: Shrub

MORPHOLOGY:

Leaves are simple alternate stipulate petiolate. Leaves with petiole 1-4 mm; blade narrowly lanceolate to oblong-lanceolate or broadly ovate, (1.7-)2.5-7.8 × (0.7-)1-3.2 cm, abaxially paler or ± glaucous; laminar glands short streaks to dots; abaxial glands dense to sparse or absent; main lateral veins (2 or)3- or 4-paired, without visible tertiary reticulation; base narrowly cordate to subcordate, apex acute to rounded. Inflorescence 1-5-flowered, from apical node, nearly round-topped; bracts deciduous, lanceolate or narrowly oblong to obovate-spatulate. Pedicels 3-16 mm.

The bark is externally brownish and internally light reddish brown in color. It occurs in the curved or sometimes flat pieces with size of 7-8 × 18-20 cm and thickness about 1.5-3 cm. It has mucilaginous taste which is followed by bitter sensation. The odor is characteristic, unpleasant and has slight astringent effect on throat. The bark shows fibrous fracture.

The bark shows number of masses of moss and fungal growth. The outer surface of bark has got numerous lenticels. Number of ringles and undulations are also seen on the outer surface, while inner surface shows presence of numerous striations. The bark is smooth and has glistering appearance due to the numerous shinning calcium oxalate crystals in sunlight. Overall the bark is compact, hard and lighter in weight. Fresh bark detached from the trunk of the tree is yellowish a turns to brown and then reddish brown on storage.

MEDICINAL USES:

- It is an ornamental plant having antibacterial activity, active against both gram-positive and gram-negative bacteria.
- Leaf and flower have strong antioxidant potential and used for liver disorders.
- Its stem is having strong antitumor, antipsychic and antiviral activities.

METHODOLOGY

A. COLLECTION AND EXTRACTION[45]

The leaves of *Hypericum mysorense* w*as* collected freshly during the month of October from Ooty, India, identified and authenticated by Dr. S Rajeswari,Department of Botany, PSG College of Arts and Science, Coimbatore comparing with the voucher specimen present in the herbarium. The collected plant, *Hypericum mysorense* was extracted by continuous hot percolation (soxhletation). 200g of powdered stem of *Hypericum mysorense* was defatted using petroleum ether. The marc obtained from the powdered plant part was successfully extracted with 250 ml of methanol by using soxhlet apparatus. The extraction was carried out for 48 hours. After extraction, the solvents were distilled out; the concentrated residues were analyzed by chemical tests.

B. EVALUATION OF METHANOLIC EXTRACT OF *Hypericum mysorense* (MeOH HMS):

The extract was evaluated for the following parameters:

- Phytochemical Analysis
- *Invitro* Antimicrobial Studies
- *Invitro* Antioxidant Property

I. PHYTOCHEMICAL ANALYSIS[46-51]

The phytochemical screening of MeOH HMS for various phytoconstituents like carbohydrate, proteins, amino acids, flavonoids, glycosides, saponins, alkaloids, steroids and tannins were done.

1. Test for carbohydrates:

a. Molisch's test

To 2-3ml of aqueous extract, added few drops of alpha naphthol solution in alcohol. Shaken and then added concentrated sulphuric acid from sides of test tube. A

brown purple ring formed at the junction of the two liquids indicates the presence of sugars.

b. Fehling's test

Mixed 1ml Fehling's solution A and 1ml of Fehling's solution B and boiled for 1 minute, added equal volume of test solution and heated on boiling water bath for 5-10 mins. Formation of brick red precipitate confirms the presence of sugars.

c. Benedict's test

Mixed equal volume of Benedict's reagent and test solution in a test tube, heated in boiling water bath for 5 mins. Formation of brick red precipitate confirms the presence of sugars.

d. Barfoed's test

Mixed equal volume of Barfoed's reagent and test solution. Heated for 1-2 mins in boiling water bath and cooled. An orange red precipitate confirms the presence of sugars.

2. **Test for glycosides**:

Heated on a water bath and the hydrolysate was subjected to Legal, Keller Killiani, Borntrager's and modified Borntrager's test to detect the presence of glycosides.

a. Legal test

To the hydrolysate, 1ml of pyridine and few drops of sodium nitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. A blood red colour indicates the presence of glycosides.

b. Borntrager's test

The hydrolysate was treated with chloroform and the chloroform layer was separated. To this, equal quantity of dilute ammonia solution was added. A light pink colour at the interface between two liquids indicates the presence of glycosides

3. **Test for alkaloids**:

a. Mayer's test

Methanolic extract was treated with drops of hydrochloric acid and filtered. The filtrate was treated with Mayer's reagent. Yellowish buff colour indicates the presence of alkaloids.

b. Dragendroff's test

Methanolic extract was treated with few drops of Dragendroff's reagent. Orange red precipitate indicates the presence of alkaloids.

c. Wagner's test

Methanolic extract was treated with drops of hydrochloric acid and filtered. The filtrate was treated with Wagner's reagent. Reddish brown precipitate indicates the presence of alkaloids.

d. Hager's test

Methanolic extract was treated with drops of hydrochloric acid and filtered. The filtrate was treated with Hager's reagent. Reddish brown colour indicates the presence of alkaloids.

4. Test for flavonoids

a. A small quantity of solvent free methanolic extract was dissolved in alcohol separately and it was hydrolyzed with 10% sulphuric acid and cooled. Then it was extracted with diethyl ether and divided in to three portions in two separate test tubes for each extract. 1ml of dilute sodium carbonate, l ml of 0.1M sodium hydroxide and 1ml of diluted ammonia solution were added to the first and second test tubes respectively. In each test tube, development of yellow colour demonstrated the presence of flavonoids.

b. Ferric chloride test:

To a small quantity of the alcohol solution of extract few drops of neutral ferric chloride solution were added. Formation of blackish red colour demonstrated the presence of flavonoids.

c. Shinoda's test

To the alcoholic solution of extract a small piece of magnesium ribbon and few drops of concentrated hydrochloric acid were added and heated, a magenta colour indicates the presence of flavonoids in methanol extract.

5. **Test for proteins**:

a. Biuret test

The extract was treated with equal volume of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution. Pink or purple colour indicates the presence of proteins.

b. Millon's test

To the extract, few drops of Millon's reagent was added and heated. Appearance of red colour indicates the presence of proteins and free amino acids.

c. Ninhydrin test

A small quantity of extract solution was boiled with 0.2% solution of Ninhydrin. Blue colour indicates the presence of free amino acids.

6. Test for tannins:

Small quantity of the extract was dissolved in distilled water, filtered and tested for the presence of phenolic compounds and tannins using the following reagents:

a. With dilute ferric chloride solution (5%) - development of greenish black coloration indicates the presence of tannins.

b. With 10% lead acetate solution - development of yellow colour precipitate indicates the presence of tannins.

c. With 10% aqueous potassium dichromate solution - development of yellowish brown precipitate indicates the presence of tannins.

7. Test for saponins:

a. Foam test

To the extract, 20ml of distilled water was added and agitated in a graduated cylinder for 15 mins. The formation of about 1cm layer of foam indicates the presence of saponins.

8. Test for steroids and triterpenoids:

a. Libermann-Burchard reaction

Small quantities of solvent free methanol extract were separately dissolved in l ml of chloroform and lml of acetic anhydride was then added followed by 2ml of concentrated sulphuric acid. A reddish violet ring at the junction of the two layers indicates the presence of triterpenoids and steroids.

b. Salkowski's test

Concentrated sulphuric acid was added to a chloroform solution of the extracts (l0mg of extract in lml of chloroform),a reddish blue colour in the chloroform layer and green fluorescence in acid layer, suggests the presence of steroids.

II. *INVITRO*- ANTIMICROBIAL STUDIES [52-53]

Antimicrobial testing is done for any organism that contributes to an infectious disease warranting antimicrobial therapy. These studies are done by two methods:

- Zone of inhibition (disc diffusion method)

DISC DIFFUSION METHOD

PRINCIPLE:

When a filter paper disc impregnated with a chemical is placed in medium containing agar, the chemical will diffuse from the disc into the agar. The diffusion places the chemical in the agar around the disc. The area of chemical infiltration around the disc is determined by the solubility of the chemical and its molecular size. An organism placed on the agar fails to grow in the area around the disc if it is susceptible to

the chemical. This area around the disc where no growth is observed is known as a "zone of inhibition".

The drug potency is established by comparing the inhibition produced by the test compound with that produced by known concentration of reference standard. When performing these tests certain things are held constant so only the size of the zone of inhibition is variable. Conditions that must be constant from test to test include the agar used, the amount of organism used, the concentration of chemical used, and incubation conditions (time, temperature and atmosphere).

A. ANTI BACTERIAL SCREENING BY DISC DIFFUSION METHOD

PREPARATION OF INOCULUM

The inoculums for the experiment were prepared in fresh Nutrient broth from preserved slant culture. The inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards. The turbidity of the culture may be adjusted by the addition of sterile saline or broth

PREPARATION OF STERILE SWABS

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or dry heat (only for wooden swabs) by packing the swabs in culture tubes, papers or tins etc.

STERILIZATION OF FORCEPS

Sterilize forceps by dipping in alcohol and burning off the alcohol

PROCEDURE

The standardized inoculums is inoculated in the plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing by pressing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60 □ C after each application.

Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed.

Each petri dish is divided into 3 parts, in 2 parts extract discs such as control and MeOH HMS (100mcg) discs (discs are soaked overnight in extract solution) and one quadrant for standard Ciprofloxacin 10mcg, are placed in each quadrant with the help of sterile forceps. The petri dishes are placed in the refrigerator at 4 º C or at room temperature for 1 hour for diffusion. Incubate at 37 º C for 24 hours. Observe the zone of inhibition produced by different samples. Measure it using a scale or divider or venire calipers and record the average of two diameters of each zone of inhibition.

Table I: List of bacteria used for the study

Sl.no	Gram positive bacteria	Gram negative bacteria
1.	*Bacillus subtilus*	*Escherichia coli*
2.	*Staphylococcus aureus*	*Salmonella paratyphi*

B. ANTI FUNGAL SCREENING BY DISC DIFFUSION METHOD

PREPARATION OF INOCULUM

The inoculums for the experiment were prepared in fresh sabouraud's broth from preserved slant culture. The inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards. The turbidity of the culture may be adjusted by the addition of sterile saline or broth.

PREPARATION OF STERILE SWABS

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or dry heat (only for wooden swabs) by packing the swabs in culture tubes, papers or tins etc.

STERILIZATION OF FORCEPS

Sterilize forceps by dipping in alcohol and burning off the alcohol.

PROCEDURE

The standardized inoculums is inoculated in the plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing by pressing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times rotating the plate through an angle of 60 º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed.

Each petri dish is divided into 3 parts , in 2 parts extract discs such as Control and MeOH HMS (100mcg) discs ,(discs are soaked overnight in extract solution) and one quadrant for standard clotrimazole 10mcg, are placed in each quadrant with the help of sterile forceps. Then petri dishes are placed in the refrigerator at 4 º C or at room temperature for 1 hour for diffusion. Incubate at room temperature for 24 - 48 hours. Observe the zone of inhibition produced by different samples. Measure it using a scale or divider or venire calipers and record the average of two diameters of each zone of inhibition.

Table II: List of fungus used for the study

Sl.no	Fungi
1.	*Candida albicans*
2.	*Aspergillus niger*

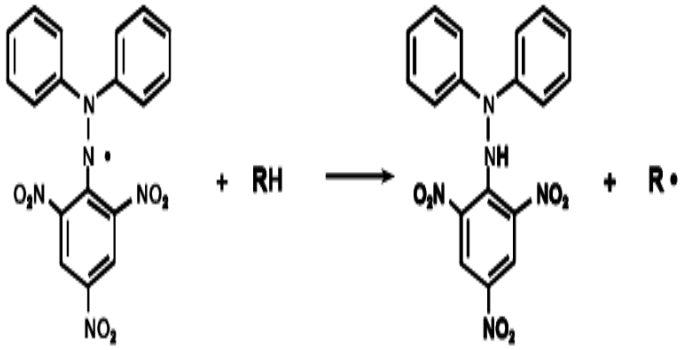
III.*INVITRO*-ANTIOXIDANT STUDIES [54-56]

A. DPPH RADICAL SCAVENGING ASSAY

The molecule of 1, 1-diphenyl-2-picryl-hydrazyl (α, α-diphenyl-β-picryl hydrozyl) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet color, characterized by an absorption band in ethanol/methanol solution centered at about 520 nm. When a solution of DPPH is mixed with that of a substance

that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (although there would be expected to be a residual pale yellow color from the picryl group still present). Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is Z•+ AH = ZH + A•

Radical scavenging activity of methanolic extract against stable DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate), was determined spectrophotometrically. The changes in color (from deep—violet to light—yellow) were measured at 517nm on a UV/visible light spectrophotometer.



Radical Form Reduced Form

0.3mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 1ml of various concentrations of sample and the reference compound (10, 20, 30, 40 and 50 µg/ml), were shaken vigorously and left to stand in the dark at room temperature for 30 min and then absorbance was measured at 517 nm. A control reaction was carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Antiradical activity was expressed as inhibition percentage (I %) and calculated using the following equation:

$$\textbf{Inhibition percentage (I \%)} = (Abs_{control} - Abs_{sample}/Abs_{control}) \times 100$$

The previous equation the term Abs_{sample} was substituted with (Abs_{sample}-Abs_{blank}). Different sample concentrations were used in order to obtain calibration curves and to

calculate the EC50 values (EC50: concentration required to obtain a 50% radical scavenging activity).

B. FERRIC REDUCING ABILITY OF PLASMA/FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

The method is based on the reduction of a ferric 2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ).

Reagent preparation:

Reagents included 300 mmol/l acetate buffer, pH 3.6 [3.1 g $C_2H_3NaO_2.3H_2O$ (sodium acetate trihydrate) and 16 ml $C_2H_4O_2$ (acetic acid) per liter of buffer solution]; 10 mmol/l TPTZ (2, 4, 6-tripyridyl-s-triazine) in 40 mmol/l HCl; 20 mmol/l $FeCl_3.6H_2O$ (ferric chloride hexahydrate). Working FRAP reagent was prepared as required by mixing 25ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml$FeCl_3.6H_2O$ solution in the ratio 10:1:1. All reagents were prepared freshly.

Samples:

To 900 µl of FRAP reagent add different concentrations of sample solution (10, 20, 30, 40 and 50 µg/ml) and the final volume was made up to 1ml.

The increase in absorbance at 593 nm was measured at 4 min. $FeSO_4.7H_2O$ was used as a standard. FRAP value was expressed as mmol/100 g on dry weight basis using the calibration curve of Fe^{2+}.

C. PREPARATION OF SEMI-SOLID FORMULATION:

I. FORMULATION OF OINTMENT:

Preparation of Ointment Base(Emulsifying Ointment)

Emulsifying wax	- 50g
White soft paraffin	- 20g
Liquid paraffin	- 30g

Procedure[56]

Required quantities of emulsifying wax, liquid paraffin and white soft paraffin were weighed and melted. To this, adequate quantity of methanolic extract of plant was added and stirred well until a homogeneous mass were obtained. The composition of the prepared herbal ointment (F1) is listed in Table III.

Table III: Composition of Ointment

SL.NO	INGREDIENTS	F1
1	Methanolic extract of *Hypericum mysorense* stem	2gm
2	Emulsifying Ointment	q.s to 100gm

D.EVALUATION OF SEMI-SOLID FORMULATION:

I. EVALUATION OF OINTMENT[58]:

PHYSIOCHEMICAL EVALUATION:

a. Colour:

The colour of the formulation was identified by visual examination.

b. Loss on drying*:*

Loss on drying was determined by placing ointment in petridish on water bath and dried for 105^0C

Percentage Loss on Drying = (Weight- Molecular Weight)/Weight × 100

c. pH:

The pH of the formulation was determined by using Digital pH meter. One gram of ointment was dissolved in 100 ml of distilled water and stored for two

hours. The measurement of pH was done in triplicate and average values were taken.

d. Viscosity:

The viscosity of the formulation was determined as such without dilution by Brookefield viscometer(model LV-DV-II, Helipath-spindle typeS-96).

e. Melting Point:

Melting Point was obtained by using melting point apparatus wherein required quantity of sample was filled in a capillary and placed in melting point apparatus and noted the temperature of melting.

f. Spreadability:

Spreadability is a term expressed to denote the extent of area to which the ointments readily spreads on application to skin or affected part. A special apparatus has been designed by Multimer to study the spreadability of formulations. The spreadability was expressed in terms of times in seconds taken by two slides to slip off from ointment and placed in between the slides under the direction of certain load. Lesser the time taken for separation of two slides, result the better spread ability. Spreadability was calculated by using the formula.

$$S = (M.L/T)$$

where, S = Spreadability

M = Weight tied to upper slide

L = Length of glass slides and

T = Time taken to separate completely from each other.

g. Extrudability

A simple method was adopted for this study. The formulation was filled in the collapsible tubes after the ointments were set in the container. The extrudability was determined in terms of weight in grams required to extrude a 0.5 cm of ribbon of ointment in 10 second.

h. HPTLC Analysis:

HPTLC analysis was conducted in KMCH College of Pharmacy.The study was carried out using HPTLC plate silica gel 60F 254(10×10cm) using toluene:ethyl acetate:formic acid:methanol (3:6:1.6:0.4) as mobile phase. The percentage of active ingredient(quercetin) present in the ointment containing 2% methanolic extract was compared with that of standard.

i. *In-vitro* drug diffusion studies[59]

In-vitro diffusion studies were performed by using franz diffusion cell with a receptor compartment capacity of 25 ml. The synthetic cellophane membrane was mounted between donor and receptor compartment of the diffusion cell. The formulated ointment was placed over the drug release membrane and the receptor compartment of the diffusion cell was filled with phosphate buffer pH 7.4. The whole assembly was fixed on a magnetic stirrer, and the solution in the receptor compartment was constantly and continuously stirred using magnetic beads at 50 rpm. The sample of 2ml were withdrawn at time interval of 30,60,90,120,150 and 180mins, then analyzed for spectrophotomerically at 369 nm.

j. Release Kinetics:

To study the release kinetics, data obtained from *in vitro* drug release studies were plotted. In various kinetic models: zero order (Equation 1) as cumulative amount of drug released Vs. time, first order (Equation 2) as log cumulative percentage of drug remaining vs. time, And Higuchi's model (Equation 3) as cumulative percentage of drug released vs. squareroot of time.

$$C = K_0 t \qquad (1)$$

Where,

K_0: is the zero-order rate constant expressed in units of concentration/time

t: is the time in hours.

A graph of concentration vs time would yield a straight line with a slope equal to K_0 and intercept the origin of the axis.

$$\mathbf{LogC = LogCo - kt/2:303} \qquad \mathbf{(2)}$$

Where, $\mathbf{C_0}$: is the initial concentration of drug,

K: is the first order constant, and t is the time.

$$\mathbf{Q = Kt^{1/2}} \qquad \mathbf{(3)}$$

Where **K:** is the constant reflecting the design variables of the system

t: is the time in hours.

Hence, drug release rate is proportional to the reciprocal of the square root of time.

k. Stability Studies:

The stability studies were carried out in formulation at different temperature conditions (4,25 and 40°C) for 3 months. All the evaluation parameters i.e. pH, viscosity, spreadability, extrudability and drug content were studied at different time intervals i.e. 15th,30th, 60th and 90th day

E. PHARMACOLOGICAL EVALUATION OF THE FORMULATED OINTMENT:

ACUTE SKIN IRRITATION STUDY

The primary skin irritation test was performed on albino rats and weighing about 150-200gm. The animals were maintained on standard animal feed and had free access to water *ad libitum*. The animals were kept under standard laboratory condition. The total mass was divided into four batches, each batch containing six animals. Two batches of each were used for control and test. Dorsal hairs at the back of the rats were clipped off one day prior to the commencement of the study. Animals showing normal skin texture were housed individually in cages with meshes to avoid contact with the bedding. 50mg of the each formulation of different concentrations were applied over one square centimeter area of intact and abraded skin to different animals. Aqueous solution of 0.8% formalin was applied as standard irritant. The animals were observed for seven days for any signs of oedema and erythema.

WOUND HEALING ACTIVITY[60]

Animals:

Wister rat of male sex, weighing 150-200g were used. All animals were obtained from KMCH College of Pharmacy. All animals were housed for at least one week in the laboratory animal room prior to testing. The selected animals were housed in polypropylene cages in standard environmental conditions (20-25°c). Fed with standard rodent diet and water as libitum. The experiments on animals were conducted in accordance with the international accepted principles for laboratory animal use and the experimental protocols duly approved by the institutional ethical committee ,KMCH college of pharmacy, Coimbatore, Reg No. 685/Po/02/a/CPCSEA.Dt:21st Aug 2002, IAEC No: KMCRET/M.Pharm/9/2013-14

Chemicals and drugs used:

- Anesthetic ether
- Povidone iodine ointment.

EXCISION WOUND MODEL[61]:

Table IV: Experimental design:

Wister rats weighing around 150-200g were used for this study. They were divided into 4 groups consisting of 6 rats each.

Table IV: Experimental Design (Excision Wound Model)

GROUP	TREATMENT : DOSE AND ROUTE OF ADMINISTRATION	NO OF ANIMALS
1	Group treated with simple ointment base	6
2	Control group with only wound	6
3	Standard group treated with povidone iodine ointment	6
4	Ointment containing 2% MeOH HMS	6

WOUND CREATION AND EVALUATION:

Four groups of animals containing six in each group were anaesthetised by open mask method with anaesthetic ether. The rats were depilated on the back and predetermined area of about 1.5cm (width) x 0.2cm (depth) was made on depilated ethanol-sterilized dorsal thoracic region of rats.Rats wounds were left undressed to the open environment. Wound were inspected and photographed.This model was used to monitor wound contraction and epithelization time. The progressive changes in wound area were monitored planimetrically by tracing the wound margin on a graph paper every alternate day. The changes with healing of wound, ie the measurement of wound area on graph paper were expressed as unit (mm^2) wound contraction was expressed as percentage reduction of original wound size.

WOUND CONTRACTION ASSESSMENT BY PLANIMETRY:

Procedure:

The contour of the individual wounds of both control and experimental animals was periodically measured using transparent graph sheet and the rate of healing was calculated and expressed as percentage contraction .The following formula was used to calculate the percentage of wound contraction.

$$\text{Wound contraction \%} = \frac{\text{wound area day 0-wound area day n}}{\text{Wound area day 0}} \times 100$$

PERIOD OF HEALING

Falling of scab, leaving no raw wound behind, was taken as the point of complete epithelization and the days required for this were as period of epithelization.

HISTOPATHOLOGICAL STUDY

The healing tissues obtained on the 3^{rd} day and 9^{th} day from all four groups of animals of the excision wound model were processed for histological study to determine the pattern of lay-down for collagen. The amount of collagen was quantified using Vangeison stain.

RESULTS AND DISCUSSION

A. EVALUATION OF Methanolic Extract of *Hypericum mysorense*(MeOH .HMS):

I. PHYTOCHEMICAL ANALYSIS:

The collected plant, *Hypericum mysorense* was dried, extracted with methanol and was subjected to preliminary phytochemical screening. Phytochemical screening showed the presence of steroids, alkaloids, flavonoids, saponins and tannins in the methanolic extracts.

Table V: Result of Phytochemical Evaluation

CONSTITUENTS	INFERENCE
Carbohydrates and reducing sugars	Absent
Glycosides	Absent
Alkaloids	Present
Flavanoids	Present
Proteins	Absent
Tannins	Present
Saponins	Present
Steroids	Present

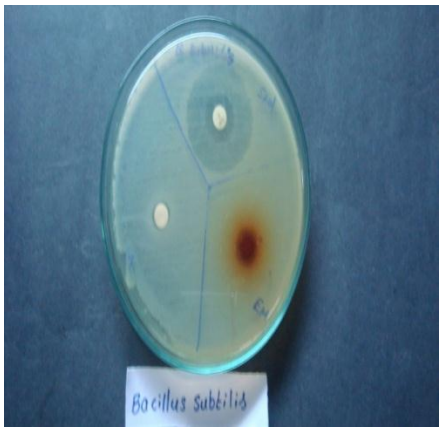
II. *INVITRO* ANTIMICROBIAL STUDIES:

a. Antibacterial Screening by Disc Diffusion Method:

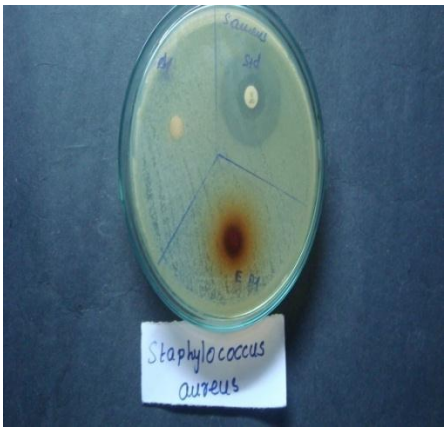
The methanolic extract was screened against various gram positive organisms like *Staphylococcus aureus* and *Bacillus subtilis* and various gram negative organisms like *Escherichia coli* and *Salmonella paratyphi.* The spectrum activity of the extract was

compared with ciprofloxacin as standard. From the table VI and figure 3, 4 and 5 it is clear that the extract possess antibacterial against selected microbial species such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Salmonella paratyphi* which is comparable with that of the standard, Ciprofloxacin.

Figure 3: Zone of inhibition against Gram positive bacteria

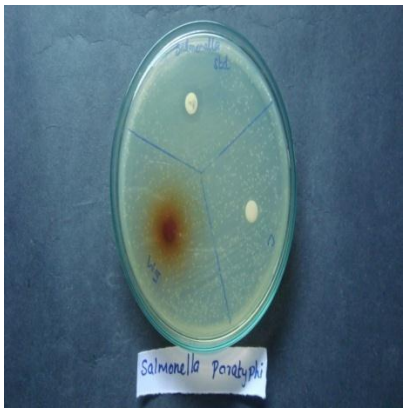


Bacillus subtilis

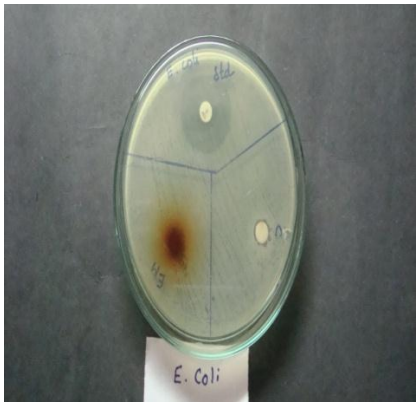


Staphylococcus aureus

Figure 4: Zone of inhibition against Gram negative bacteria



Salmonella paratyphi



Escherichia coli

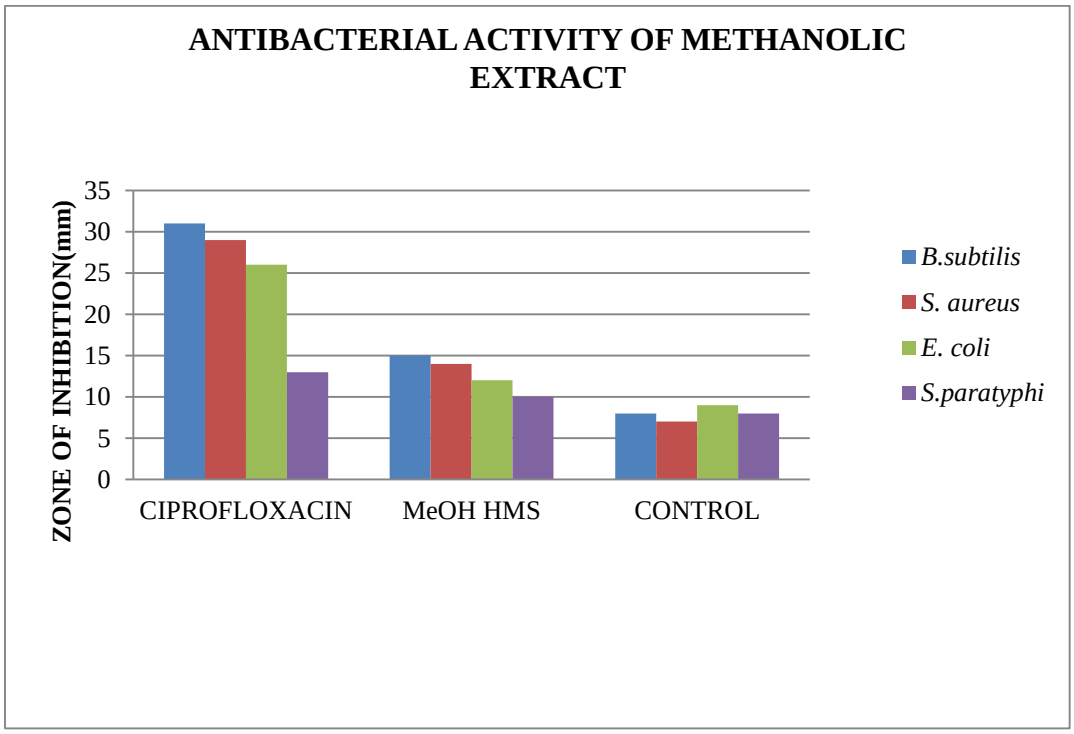
The observations are tabulated in table VI

Table VI: Antibacterial Screening by Disc Diffusion Method

Extract Used	Diameter of Zone of inhibition (mm)			
	Gram +ve Bacteria		Gram -ve Bacteria	
	B.subtilis	*S.aureus*	*E. coli*	*S.paratyphi*
Ciprofloxacin	31	29	26	13
MeOH.HMS	15	14	12	10
Control	8	7	9	8

MeOH .HMS- Methanolic Extract of *Hypericum mysorense* stem

Figure 5: Graphical Representation of Antibacterial Activity of Methanolic Extract



b. Antifungal Screening by Disc Diffusion Method:

The methanolic extract was screened against various fungus like *Candida albicans* and *Aspergillus niger*. The spectrum activity of the extract was compared with ciprofloxacin as standard. From the table VII and figure 6 and 7 it is clear that the extract possess antifungal against selected microbial species such as *Candida albicans* and *Aspergillus niger* which is comparable with that of the standard, Clotrimazole.

Figure 6: Zone of inhibition against various fungus

Aspergillus niger ***Candida albicans***

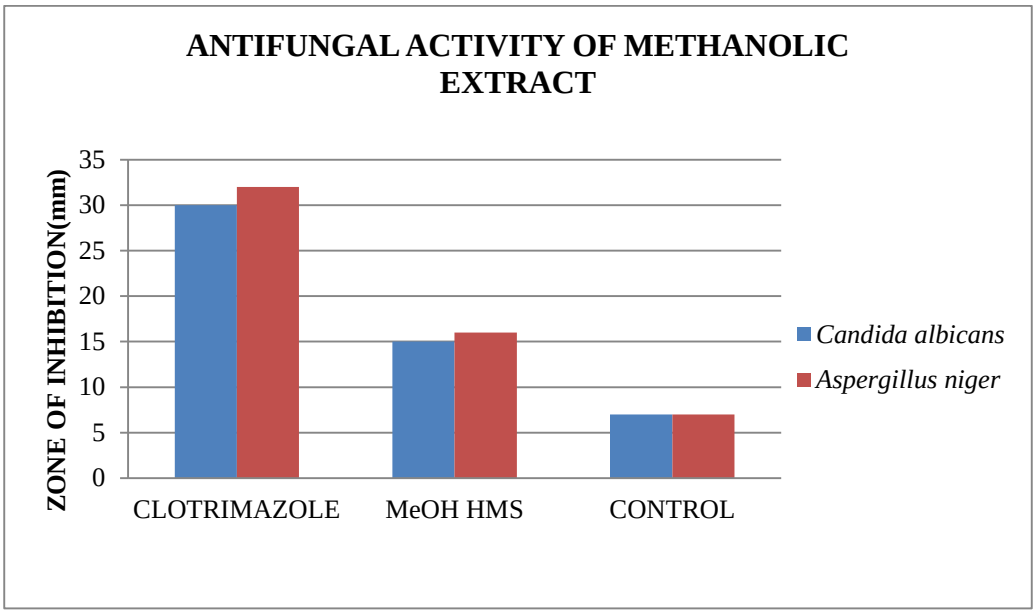
The observations are tabulated in Table VII

Table VII: Antifungal Screening by Disc Diffusion Method

Extract Used	**Diameter of Zone of Inhibition (mm)**	
	Candida albicans	***Aspergillus niger***
Clotrimazole	30	32
MeOH. HMS	15	16
Control	7	7

MeOH .HMS- Methanolic Extract of *Hypericum mysorense* stem

Figure 7: Graphical Representation of Antifungal Activity of Methanolic Extract



III. *INVITRO* ANTIOXIDANT STUDIES:

In-vitro anti oxidant activity was carried out by using the extract against DPPH and FRAP assay where ascorbic acid used as standard**.** The results showed that the extract possess anti oxidant activity.

The DPPH radicals absorb at 520 nm and this absorption is inhibited in the presence of antioxidants. This reduction in absorbance is related to the antiradical efficiency of the extract. The DPPH antiradical efficiency values of the extract analyzed in this study are presented in the Table VIII and figure 8.

The effect of methanolic extract on Fe^{3+} - Fe^{2+} transformation investigated, the reducing capacity of extract, may serve as a significant indicator of its potential antioxidant activity. Table IX and figure 9 shows the reductive effect of extract.

The antioxidant property of these studies may also be due to presence of phenol and flavonoid in our plant extract.

Table VIII: DPPH Assay

CONC(µg/ml)	% INHIBITION	
	MeOH. HMS	ASCORBIC ACID
5	43.22±0.15	49.76±0.16
10	53.07±0.17	53.08±0.17
15	65.77±0.16	69.47±0.18
20	78.33±0.15	88.42±0.14
25	95.33±0.13	98.75±0.13
EC 50	9.4±0.13	9.4±0.15

Data is expressed as Mean ± SEM

Figure 8-DPPH Assay

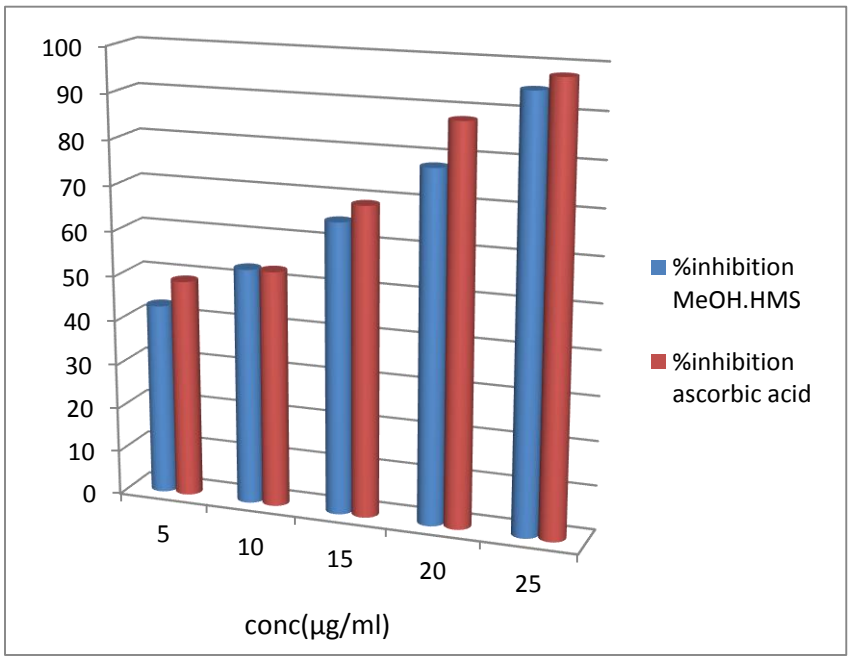
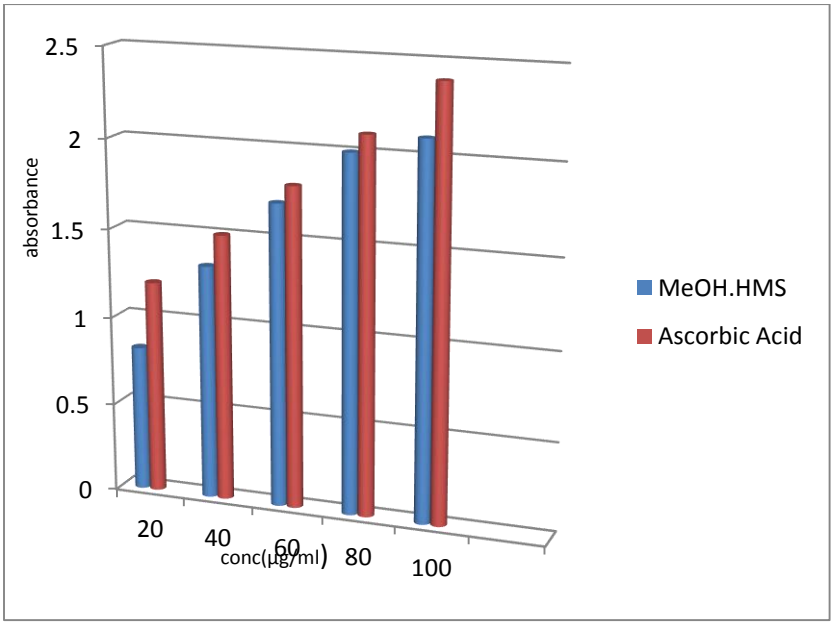


Table IX: FRAP assay

CONC. (µg/ml)	ABSORBANCE	
	MeOH .HMS	ASCORBIC ACID
20	0.8287	1.2788
40	1.3264	1.5477
60	1.7279	1.8804
80	2.0407	2.1987
100	2.195	2.4273

Figure 9-FRAP Assay



C. PREPARATION OF SEMI-SOLID FORMULATION:

The extract was formulated as an ointment with 2%w/w of the methanolic extract using simple ointment base .The formulated ointment was then subjected to various evaluation studies.

D. EVALUATION OF SEMI-SOLID FORMULATION:

1. EVALUATION OF OINTMENT:

PHYSIOCHEMICAL EVALUATION:

The formulated ointment was evaluated for various physicochemical parameters. The colour of the ointment was dark green with characteristic odour. The physicochemical parameters observed are recorded in Table X. The results of viscosity and melting point are appreciable for ointment formulation. The result clearly indicated that the ointment showed good spreadability and extrudability. Spreadability expressed to denote of area to which the prepared formulations readily spread on application to skin and the extrudability is a measure of removal of the formulation through orifice of the container. The ointment showed a pH that lie in the normal pH range of human skin and is easily diffusible.

Table X: Result of Physiochemical Evaluation of Ointment

Parameters	Observation
Colour	Dark green
Odour	Characteristic
Loss on Drying	37%W/W
pH	5.8
Viscosity	16.05cps
Melting Point	38°C
Spreadability(seconds)	12
Extrudability	14.2g

HPTLC ANALYSIS:

Table XI: Result of HPTLC Analaysis

Sl.No	Samples	Maximum Rf	% Area
1	HMME	0.84	7.81
2	Ointment containing 2% MeOH.HMS	0.83	9.85
3	Quercetin	0.83	87.89

HMME- Hypericum mysorense methanolic extract

From the above result, it can be concluded that the methanolic extract of *Hypericum mysorense* and the prepared ointment formulation contain quercetin as active ingredient.

IN-VITRO DIFFUSION STUDIES OF OINTMENT:

CALIBRATION CURVE OF QUERCETIN:

Quercetin (50mg) was weighed accurately and dissolved in 50ml of methanol in 50ml volumetric flask (stock –I). from this stock –I 1 ml solution was withdrawn and diluted to 10ml (Stock –II). From the stock –II solution serial dilutions were prepared by taking 0.2, 0.4, 0.6, 0.8, 1.0 and making the volume up to 10ml, which gives the concentrations of 2,4,6,8,10 µg/ml and the absorbance was noted at 369nm.

Table XII: Caliberation Curve of Quercetin

CONCENTRATION(µg/ml)	ABSORBANCE
0	0
2	0.22
4	0.456
6	0.643
8	0.908
10	1.142

Figure 10: Caliberation curve of Quercetin

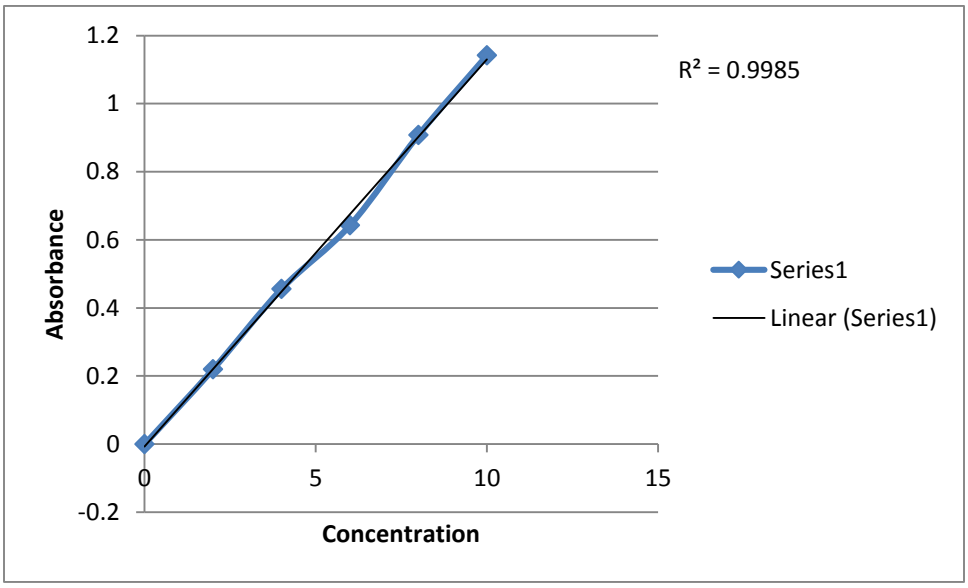
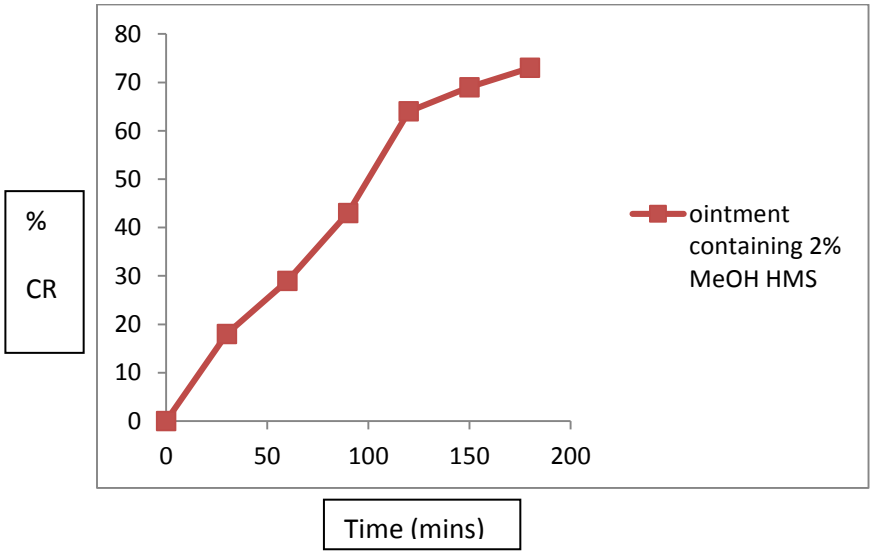


Table XIII: Result of *in-vitro* diffusion studies

Time(mins)	% Cumulative Release
	Ointment containing 2% MeOH HMS
30	18
60	29
90	43
120	64
150	69
180	73

Figure 11: Figure of *in-vitro* diffusion study



KINETIC ANALYSIS:

The *in-vitro* drug release studies were subjected to kinetic analysis by plotting various kinetic equations like zero order, first order, Higuchi plot. They were also subjected for peppas plot in order to find out the mechanism of release from the prepared patches . The kinetic analysis data of the formulations were shown in table no XIII.

The data suggest that the formulation fit into first order equation for the release of drug that depends mostly on diffusion characterstics.

Table XIV: Release Kinetics of Formulated Ointment

Formulation	Zero Order	First Order	Higuchi	
	R^2	R^2	R^2	n
2% ointment containing MeOH.HMS	0.925	0.966	0.939	0.837

Figure 12: Zero Order Plot of Formulation

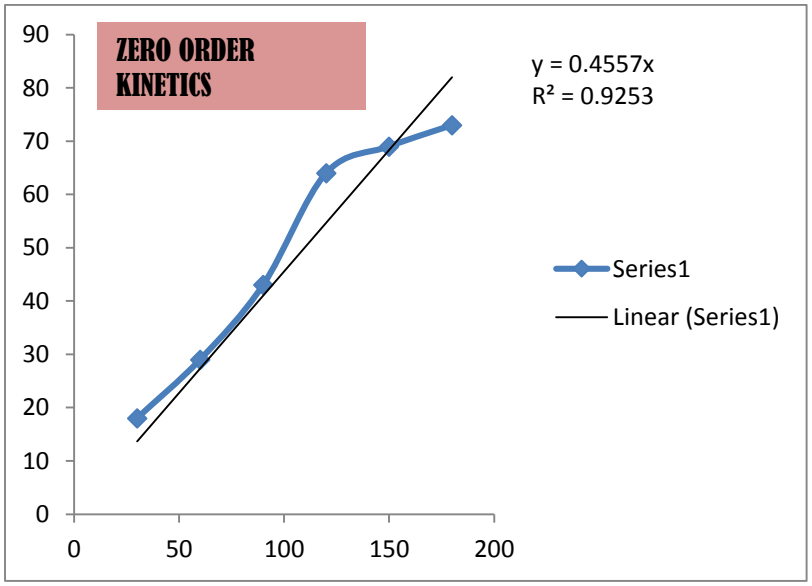


Figure 13: First Order Plot of Formulation

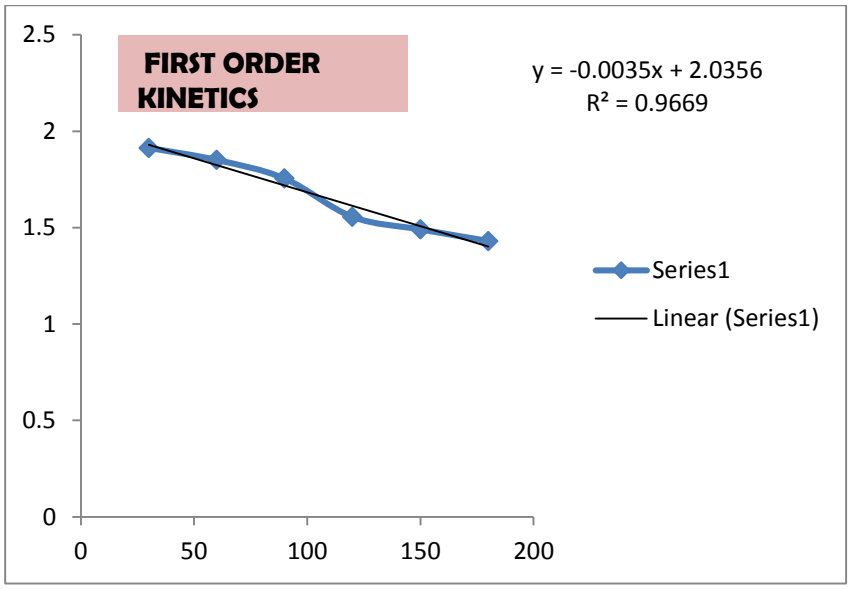
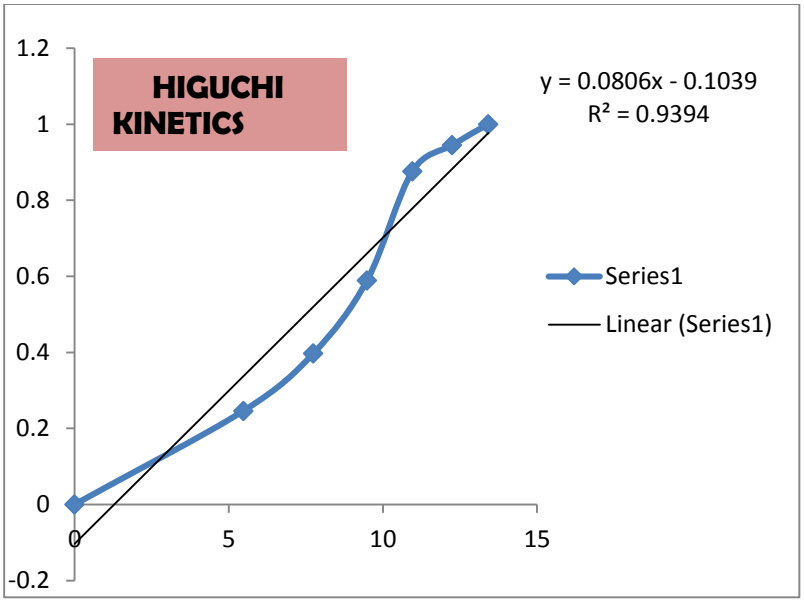


Figure 14: Higuchi Plot of Formulation



STABILITY STUDIES:

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light to establish shelf-life for the drug product and recommended storage conditions.

Ointments at the concentrations of 2%,showed no changes in pH, viscosity, spreadability, extrudability, drug content after keeping at different temperatures for 90days

Table XV: Result of Stability Studies

Sl. No	Formulation	Temp	Parameters					
			Colour	viscosity	Spreadabiliy	extrudability	MPt	Assay
1	Oint cointaining 2% extract (1st month)	4°	Dark green	20.15 cps	8	5.7g	38°	99.8%
		25°	Dark green	16.05 cps	10	13.8g	38°	
		40°	Dark green	16.01 cps	12	14.2g	38°	
2	Oint cointaining 2% extract (2nd month)	4°	Dark green	20.32 cps	7.5	5.5g	38°	98.9%
		25°	Dark green	15.98 cps	No change	No change	38°	
		40°	Dark green	15.95 cps	No change	No change	38°	
3	Oint cointaining 2% extract (3rd month)	4°	Dark green	20.3cps	7.4	5.3g	38°	98.5%
		25°	Dark green	No change	No change	No change	38°	
		40°	green	No change	No change	No change	38°	

E. PHARMACOLOGICAL EVALUATION OF THE FORMULATED OINTMENT:

WOUND HEALING ACTIVITY:

Table XVI: Percentage Wound closure

Test Compounds	0th day	3rd day	7th day	13th day	17th day	19th day
	% WC	% WC	% WC	% WC	%WC	%WC
STD	0	15.7 ±0.29	39.8 ± 0.77	81.8 ± 1.09	88.5 ± 1.25	95.5 ± 1.10
OINTMENT BASE	0	13.5 ±0.49	27 ± 0.83	52.5 ± 1.24	66.8 ± 1.24	88.2 ± 1.88
CONTROL	0	27.5 ± 0.58	23.6 ± 2.03	74.2 ± 0.99	72 ± 0.99	90.1 ± 0.99
OINTMENT CONTAINING 2%EXTRACT	0	10 ± 0.42	13.8 ± 1.48	55.4 ± 1.08	84.1 ± 1.08	96.9 ± 1.08

Results were expressed as mean (mm) ± SEM and were compared with the corresponding control group (simple ointment B.P.) by applying ANOVA test. P value was set <0.05 for all analyses**.**

Figure15: Figure showing percentage wound closure

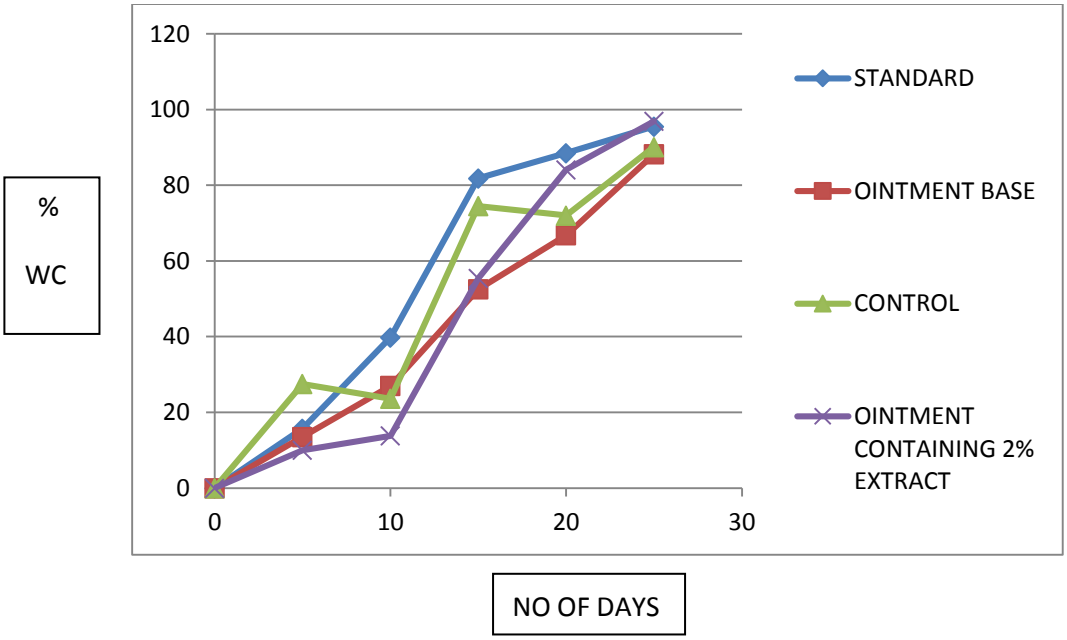


Table XVII: Figure showing percentage wound reduction

Test compounds	0 TH day	3 rd day	7 th day	13 th day	17 th day	19 th day
	%WR	%WR	%WR	%WR	%WR	%WR
STD	100±0.00	84.3±0.29	60.2±0.77	18.2±1.09	11.5±1.25	4.42±1.10
OINTMENT BASE	100±0.00	86.5±0.49	73±0.83	47.5±1.24	33.2±1.24	11.8±1.88
CONTROL	100±0.00	72.5±0.58	76.4±2.03	25.8±0.99	28±0.99	9.81±0.99
OINTMENT CONTAINING 2% EXTRACT	100±0.00	90±0.42	86.1±1.48	44.5±1.08	15.8±1.08	3.02±1.08

Results were expressed as mean (mm) ± SEM and were compared with the corresponding control group (simple ointment B.P.) by applying ANOVA test. P value was set <0.05 for all analyses

Figure 16: Figure showing percentage wound reduction

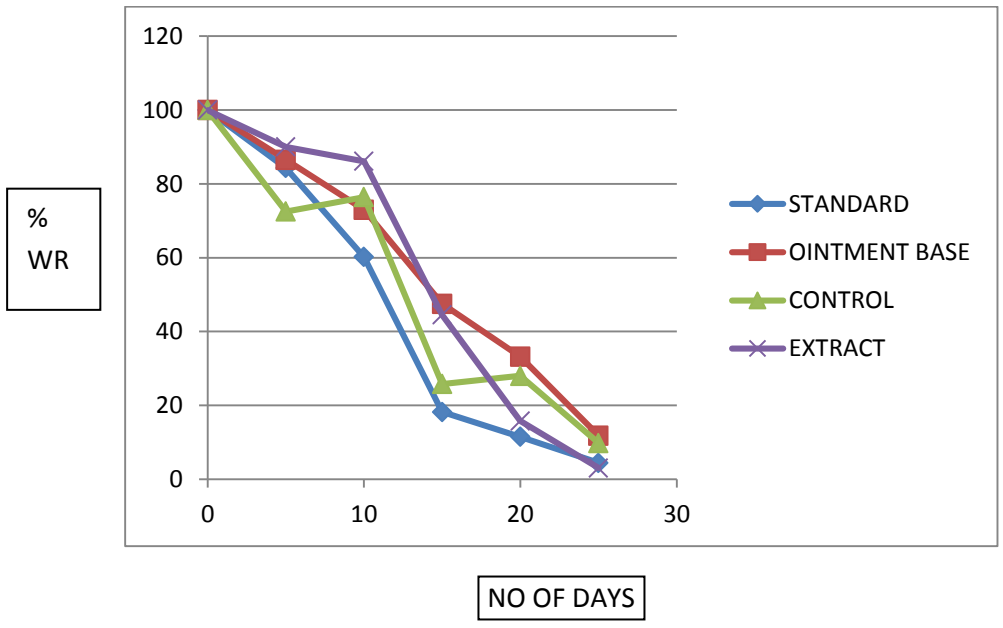


Figure 17: Figure showing the wound healing activity from 0th day — 21th day.

EXCISION WOUND ON 0TH DAY

ONLY WOUND

WOUND + OINTMENT BASE

WOUND + OINT CONTAINING 2% MeOH.HMS

WOUND + STANDARD

EXCISION WOUND ON 3RD DAY

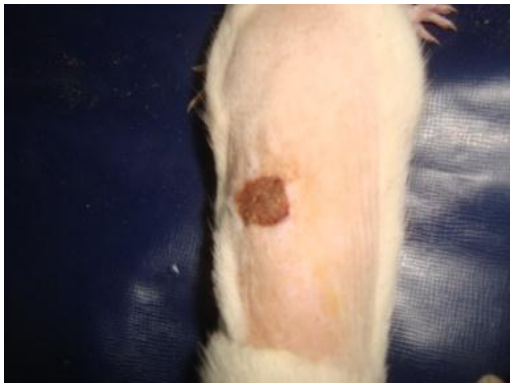
ONLY WOUND

WOUND + OINTMENT BASE

WOUND + OINT CONTAINING 2% MeOH.HMS

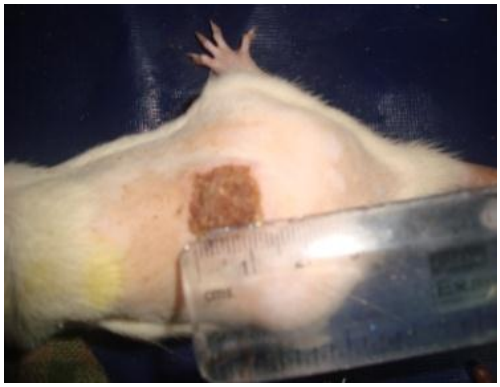
WOUND + STANDARD

EXCISION WOUND ON 5TH DAY



ONLY WOUND

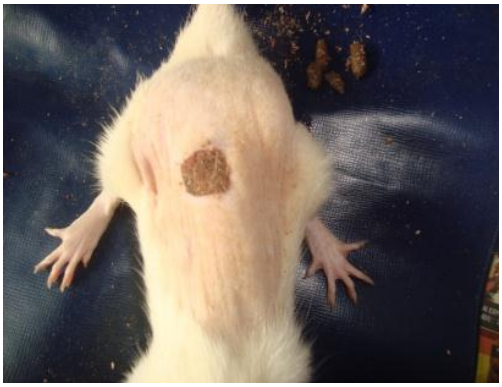
WOUND + OINTMENT BASE



WOUND + OINT CONTAINING 2% MeOH.HMS

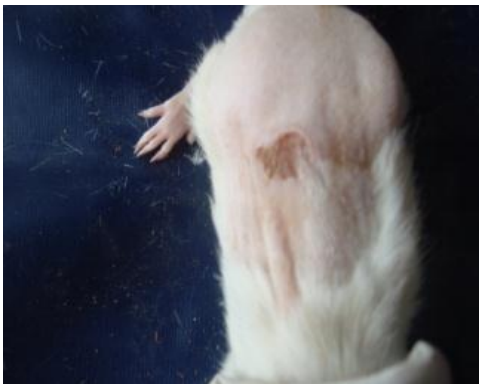
WOUND + STANDARD

EXCISION WOUND ON 7TH DAY

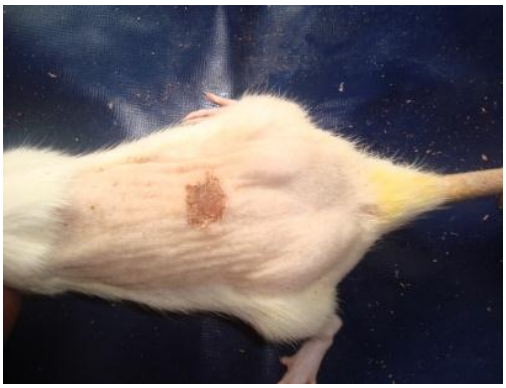


ONLY WOUND

WOUND + OINTMENT BASE



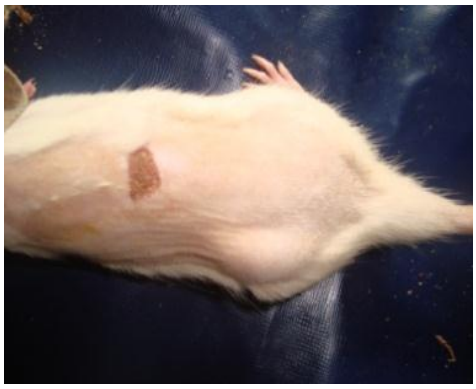
WOUND + OINT CONTAINING 2% MeOH.HMS



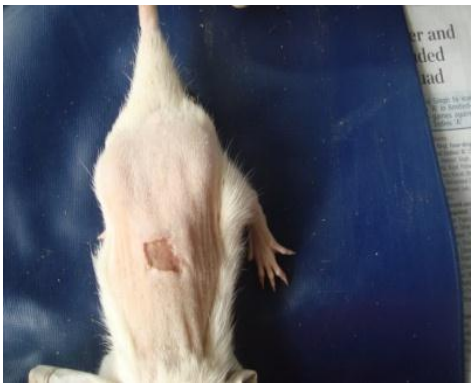
WOUND+ STANDARD

EXCISION WOUND ON 9TH DAY

ONLY WOUND



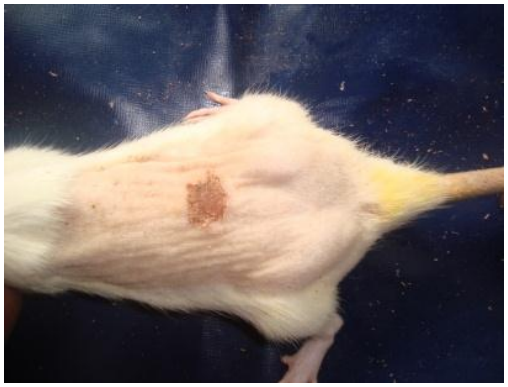
WOUND+OINTMENT BASE



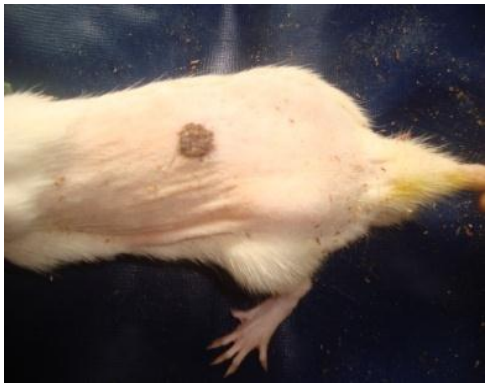
WOUND + OINT CONTAINING 2% MeOH.HMS

WOUND + STANDARD

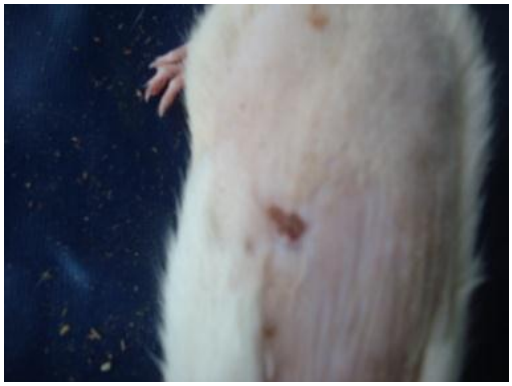
EXCISION WOUND ON 13TH DAY



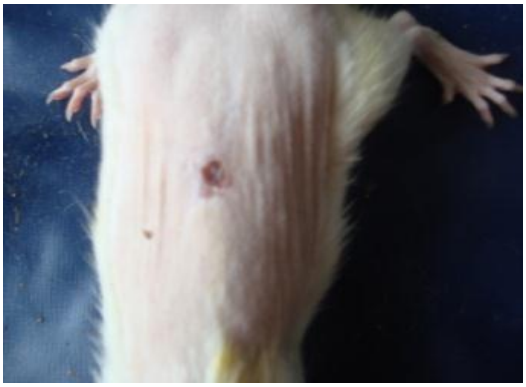
WOUND ONLY



WOUND+ OINTMENT BASE

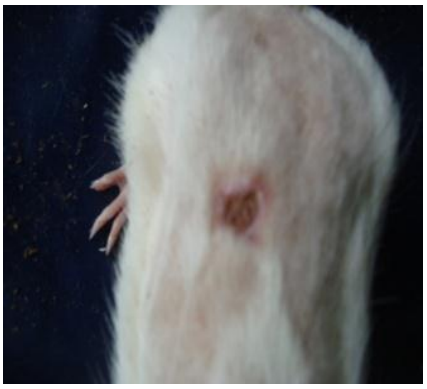


WOUND + OINT CONTAINING 2% MeOH.HMS



WOUND+ STANDARD

EXCISION WOUND ON 17TH DAY



ONLY WOUND

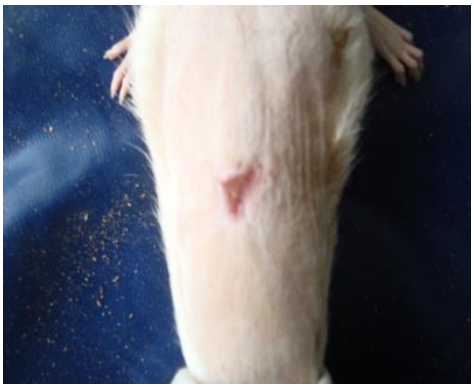
WOUND+ OINTMENT BASE

WOUND + OINT CONTAINING 2% MeOH.HMS

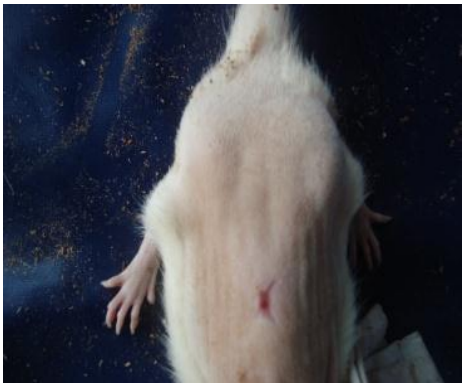
WOUND + STANDARD

EXCISION WOUND ON 19TH DAY

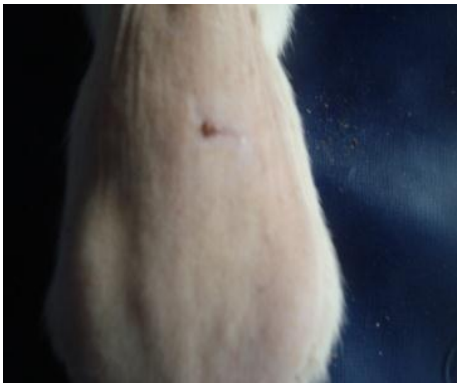
ONLY WOUND



WOUND + OINTMENT BASE



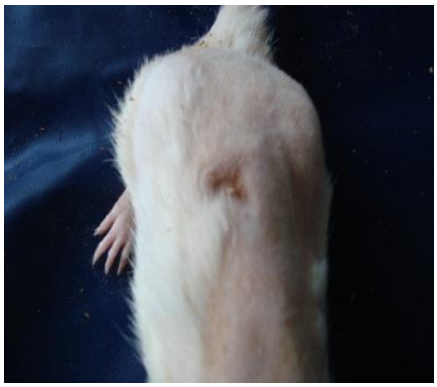
WOUND + OINT CONTAINING 2% MeOH.HMS



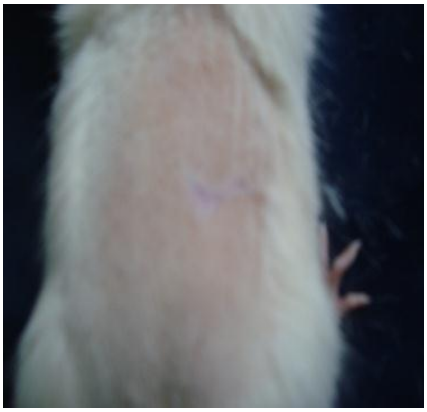
WOUND+ STANDARD

EXCISION WOUND ON- 21ST DAY

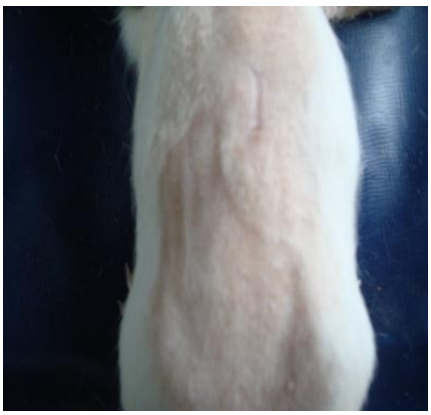
ONLY WOUND



WOUND + OINTMENT BASE



WOUND + OINT CONTAINING 2% MeOH.HMS



WOUND+ STANDARD

Histopathological studies:

The healing tissues obtained on the 3rd and 9th day from the four groups of animals of the excision wound model were processed for histological study to determine the pattern of lay-down for collagen. The amount of collagen was quantified using Vangeison stain.

Wound + Standard(3rd Day)

Gross appearance: Received multiple tiny grey white soft tissue bits altogether measuring 0.6 x 0.4 x 0.3 cms.

(AE): All embedded – one block.

Microscopic appearance: Section studied from the skin shows acute ulcer with dense chronic inflammation composed of lymphocytes and plasma cells. No granulation tissue or fibrosis noted.

WOUND + OINT CONTAINING 2% MeOH.HMS (3rd Day)

Gross appearance: Received multiple tiny grey white soft tissue bits altogether measuring 0.7 x 0.4 x 0.2 cms.

(AE): All embedded – one block.

Microscopic appearance: Section studied from the skin shows dense chronic inflammation composed of lymphocytes and plasma cells with early stage of granulation tissue. And mild fibrosis also noted.

Wound + Standard (9th Day)

Gross appearance: Received multiple tiny grey white soft tissue bits altogether measuring 1.0 x 0.5 x 0.3 cms.

(AE): All embedded – one block.

Microscopic appearance: Section studied from the skin shows acute ulcer with granulation tissue with edema. Fibroblasts and papillary proliferation and mild fibrosis are seen.

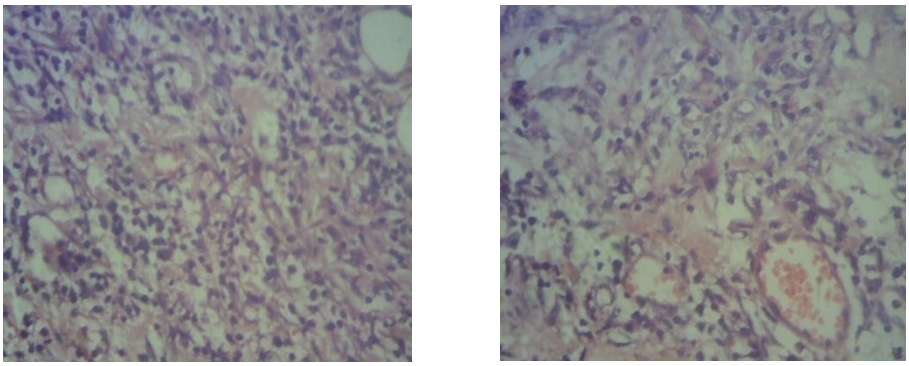
Wound + OINT CONTAINING 2% MeOH.HMS (9th Day)

Gross appearance: Received multiple tiny grey white soft tissue bits altogether measuring 0.9 x 0.4 x 0.4 cms.

(AE): All embedded – one block.

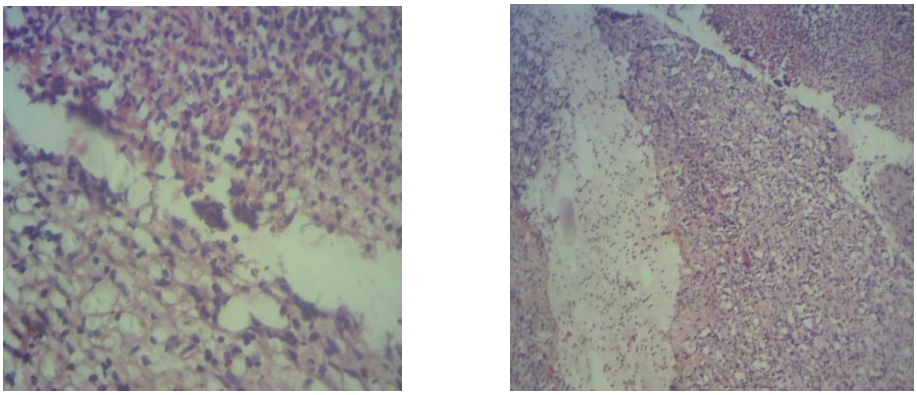
Microscopic appearance: Section studied from the skin shows granulation tissue with dense chronic inflammation along with scattered fibroblasts and proliferating papillae.

Figure 18: 3rd day microscopical view of the wound tissue



STANDARD OINT CONTAINING 2% MeOH.HMS

Figure 19: 9th day microscopical view of the wound tissue



STANDARD OINT CONTAINING 2% MeOH.HMS

INFERENCE:

The 2% concentration of the methanolic extract ointment showed significant responses in excision wound model when compared with the control group. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time, regeneration of tissues at wound site, and histopathological characteristics were comparable to those of a standard drug, Povidone Iodine ointment.

SUMMARY

Hypericum mysorense is an ornamental plant belonging to the family Hypericaceae, having antibacterial activity, against both gram-positive and gram-negative bacteria. Leaf and flower have strong antioxidant potential and used for liver disorders. Its stem is having strong antitumor, antipsychic and antiviral activities. Among the various indications where traditional herbal medicines are used, skin and skin related disorders are ranked top. Thus, the main objective of the present study is to formulate and evaluate semi-solid dosage forms of *Hypericum mysorense* for its wound healing activity.

FORMULATION:

The stem of *Hypericum mysorense* was extracted by continuous hot percolation/ soxhletation using methanol as solvent and was evaluated for its phytochemical property, *in-vitro* antimicrobial and *in-vitro* antioxidant activity. Using this methanolic extract, semi-solid dosage form (ointment) was formulated. Ointment was prepared with 2% methanolic extract using emulsifying ointment base.

EVALUATION:

The methanolic extract was evaluated for its *in-vitro* antimicrobial and antioxidant properties using disc diffusion method and DPPH, FRAP analysis respectively. The results suggested that the plant possess activity comparable to the standard, ciprofloxacin (anti-bacterial), clotrimazole(anti-fungal) and ascorbic acid (anti-oxidant). Further, the formulated ointment was evaluated for their physiochemical properties like colour, pH, viscosity, melting point, spreadability, extrudability etc. HPTLC analysis, *in-vitro* diffusion studies, release kinetics and stability studies were also conducted on the preparation. The results of *in-vitro* antimicrobial and *in-vitro* antioxidant activity added on to the wound healing activity of the extract. Hence, the ointment containing 2% Methanolic Extract of *Hypericum mysorense* stem was evaluated for its wound healing activity using excision wound model. The formulation did not produce any skin irritation for about a week when applied over the skin. Wister rats weighing around 150-200g were used for the study. They were divided into 4 groups consisting of 6 rats each. The 4

groups include standard(povidone iodine ointment), control(only wound),rats treated with ointment base and rats treated with 2% ointment. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug, povidone iodine ointment. Thereby, the ointment formulated using 2% methanolic extract of *Hypericum mysorense* was found to possess wound healing activity.

CONCLUSION

- Methanolic extract of *Hypericum mysorense* was prepared by continous hot percolation and was evaluated for its phytochemical property, *in-vitro* antimicrobial and *in-vitro* antioxidant activity.

- Using this methanolic extract, semi-solid dosage form (ointment) was formulated. Ointment were prepared with 2% methanolic extract using emulsifying ointment base and was then screened for its physiochemical property,HPTLCanalysis, *in-vitro* drug diffusion,kinetic studies and stability studies. The results of *in-vitro* antimicrobial and *in-vitro* antioxidant activity added on to the wound healing activity of the extract.

- Hence, the 2% ointment was evaluated for its wound healing activity using excision wound model. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug, povidone iodine ointment.

- Therefore, the present study concluded the wound healing activity of *Hypericum mysorense* stem at 2% concentration of the methanolic extract formulated as an ointment.

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EVALUATION OF METHANOLIC EXTRACT OF *Hypericum mysorense* OINTMENT FOR ITS WOUND HEALING ACTIVITY

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ABBREVIATIONS

- MeOH HMS — Methanolic extract of *Hypericum mysorense*
- Oint — Ointment
- MP — Melting point
- % — Percentage
- gm. — gram
- mg. — Milligram
- µg. — Micro gram
- ml. — Millilitre

- nm. Nanometer
- W/W Weight by weight
- W/V Weight by volume
- pH. Hydrogen ion concentration
- ^{o}C Degree centigrade
- RPM. Revolution per minute
- Abs. Absorbance
- Conc. Concentration
- CR Cumulative Release
- R^2 Regression coefficient

INTRODUCTION

- The majority of the populations in the developing world rely on traditional medicine for their primary healthcare needs. Herbal therapy predominates in traditional medicine as well as in complementary or alternative medicine practiced in the developed world.

- *Hypericum mysorense* is a medicinal plant having antibacterial , antioxidant, antitumor, antipsychic and antiviral activities.

- Thus, the main objective of the present study is to formulate and evaluate ointment form of *Hypericum mysorense* for its wound healing activity.

- Wound healing involves a complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix.

- The phases of normal wound healing include haemostasis, inflammation, proliferation and remodeling.

- The properties leading to wound healing includes anti-oxidant, anti-inflammatory, anti- microbial ,analgesic activity.

AIM AND OBJECTIVE

The aim of the present study is to highlight the use of *Hypericum mysorense* for the treatment of cuts and wounds as a wound healer. The study involves the formulation and evaluation of methanolic extract of *Hypericum mysorense* as a ointment form for its wound healing activity.

The objective of the study was:

- To carry out continuous hot percolation of *Hypericum mysorense* using methanol as solvent.
- To evaluate phytochemical constituents of the obtained methanolic extract of *Hypericum mysorense*.

- To evaluate the methanolic extract of *Hypericum mysorense* for its *in-vitro* antioxidant and antimicrobial property.
- To formulate methanolic extract of *Hypericum mysorense* as semi-solid dosage form(ointment).
- To evaluate the physiochemical parameter of the formulated ointment.
- To conduct HPTLC analysis, *in-vitro* diffusion studies, release kinetics and stability studies of the prepared ointment
- To assess the wound healing property of the prepared herbal ointment by excision wound model.

LITERATURE REVIEW

Sl No	Author's name	Journal Name	Objective	Conclusion
1	Pulok K Mukerjee et al	Natural Product Sciences, 6(2):73-78(2000)	Studied *in-vivo* wound healing activity of leaf extract of *Hypericum mysorense*(5%,10%) with different wound model in rats.	Both the concentrations of methanolic extract ointment showed significant response when compared with control group
2	Shanmugam Moorthy et al	Reasearch Journal of Biotechnology, Vol 5, (1),Feb (2010)	Studied the effect of ethanolic extract of *Hypericum mysorense* on anti-oxidant and anti-depressant activity.	Indicates anti-oxidant and anti-depressant effect of ethanolic extract of *Hypericum mysorense*.

Sl No	Author's Name	Journal Name	Objective	Conclusion
3	P Vijayan, C Raghu et al	Indian Journal of Medical Research, 120,July 2004,pg 24-29	Screened various plant extracts for antiviral properties by cytopathic effect inhibition assay and virus yield reduction assay.	*Hypericum mysorense* exhibited significant antiviral activity.
4	Atul Murlidhar Wahile	Thesis submitted for the degree of Doctor of Philosophy(Pharmacy) of Jadavpur University,chapter 10,11	Investigated the analgesic potential of ethyl acetate and methanolic extract of *Hypericum mysorense* through hot plate method and acetic acid induced writhing test in mice.	*Hypericum mysorense* showed analgesic activity.

PLANT PROFILE

DESCRIPTION OF PLANT'S BOTANICAL INFORMATION:

Botanical name : *Hypericum mysorense*

Family : Hypericaceae

MEDICINAL USES:

- It is an ornamental plant having antibacterial activity, active against both gram-positive and gram-negative bacteria.
- Leaf and flower have strong antioxidant potential and used for liver disorders.
- Its stem is having strong antitumor, antipsychic and antiviral activities.

METHODOLOGY

1. EXTRACTION(CONTINUOUS HOT PERCOLATION)

200 g of powdered stem of *Hypericum mysorense* was defatted using petroleum ether.

Marc obtained was extracted with 250 ml of methanol for 48 hrs by using soxhlet apparatus

Solvent is distilled out and concentrated residue is analysed by chemical test

2. *INVITRO* ANTIMICROBIAL STUDIES

- Antibacterial screening

Sl no	Organisms used		standard	test	Control
	Gram positive	Gram negative			
1	*Bacillus subtilis*	*Escherichia coli*	Ciprofloxacin 10µg	Methanolic extract of *Hypericum mysorense* stem	Plain sterile disc
2	*Staphylococcus aureus*	*Salmonella paratyphi*			

- Antifungal screening

Sl no	Organism	Standard	Test	Control
1	*Candida albicans*	Clotrimazole 10µg	Methanolic extract of *Hypericum mysorense* stem	Plain sterile disc
2	*Aspergillus niger*			

3.*INVITRO* ANTIOXIDANT STUDIES

- DPPH assay:

0.3mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 1ml of various concentrations of sample and the reference compound (10, 20, 30, 40 and 50 µg/ml), were shaken vigorously and left to stand in the dark at room temperature for 30 min and then absorbance was measured at 517 nm. A control reaction was carried out without the test sample. All the tests were performed in triplicate in order to get the mean values. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

Antiradical activity was expressed as inhibition percentage (I %) and calculated using the following equation:

Inhibition percentage (I %) = ($Abs_{control}$- Abs_{sample}/$Abs_{control}$) X 100

- FRAP assay:

To 900 µl of FRAP reagent add different concentrations of sample solution (10, 20, 30, 40 and 50 µg/ml) and the final volume was made up to 1ml.

The increase in absorbance at 593 nm was measured at 4 min. $FeSO_4.7H_2O$ was used as a standard. FRAP value was expressed as mmol/100 g on dry weight basis using the calibration curve of Fe^{2+}.

4.COMPOSITION OF OINTMENT:

SL.NO	INGREDIENTS	F1
1	Methanolic Extract of *Hypericum mysorense*	2gm
2	Emulsifying Ointment(Emulsifying wax , White soft paraffin , Liquid paraffin)	q.s to 100gm

5. EVALUATION OF OINTMENT

- Physiochemical Evaluation:

  The physiochemical properties such as colour, loss on drying, pH, viscosity, melting point, spreadability and extrudability were analysed.

- HPTLC analysis:

  Stationary phase : HPTLC plate silica gel 60F 254(10×10cm)
  Mobile phase : Toluene:ethyl acetate:formic acid:methanol (3:6:1.6:0.4)

  The percentage of active ingredient(quercetin) present in the ointment containing 2% methanolic extract was compared with that of standard.

- *Invitro* drug diffusion study(Franz diffusion cell)

Synthetic cellophane membrane was mounted between donor and receptor compartment.

The formulated ointment was placed over the drug release membrane and the receptor compartment was filled with phosphate buffer pH=7.4

The whole assembly was fixed on magnetic stirrer(50 rpm)

2ml sample withdrawn at 30,60,90,120,150 and 180 mins.

Analysed spectrophotometrically at 369 nm.

- Release Kinetics:

To study the release kinetics, data obtained from *in vitro* drug release studies were plotted. In various kinetic models: zero order (Equation 1) as cumulative amount of drug released Vs. time, first order (Equation 2) as log cumulative percentage of drug remaining vs. time, And Higuchi's model (Equation 3) as cumulative percentage of drug released vs. squareroot of time.

$$C = K_0 t \longrightarrow (1)$$

$$LogC = LogCo - kt/2.303 \longrightarrow (2)$$

$$Q = Kt^{1/2} \longrightarrow (3)$$

- Stability Studies:

The stability studies were carried out in formulation at different temperature conditions (4,25 and 40°C) for 3 months.

All the evaluation parameters i.e. pH, viscosity, melting point, spreadability and extrudability were studied at different time intervals i.e.30th, 60th and 90th day.

- Pharmacological Evaluation

Excision Wound Model

Group	Treatment:dose and route of administration	Number of animals
1	Group treated with simple ointment base	6
2	Control group with only wound	6
3	Standard group treated with povidone iodine ointment	6
4	Ointment containing 2% MeOH.HMS	6

Animals:

Species : adult Wistar rats

Weight : 150 – 200 gms

Gender : male

Number to be used : 24

Route of Induction : Topical application

Duration : 21 days

RESULTS AND DISCUSSION

1. PHYTOCHEMICAL ANALYSIS:

CONSTITUENTS	TEST	INFERENCE
Carbohydrates	Molisch's ,Fehling's, Benedict's, Barfoed's	Absent
Glycosides	Legal, Borntrager's	Absent
Alkaloids	Mayer's, Dragendroff's, Wagner's, Hager's	Present
Flavanoids	Ferric chloride, Shinoda's	Present
Proteins	Biuret, Ninhydrin, Millon's	Absent
Tannins	Ferric chloride, Lead acetate	Present
Saponins	Foam test	Present
Steroids	Liebermann- Burchard, Salkowski	Present

2. *INVITRO* ANTIMICROBIAL STUDIES

- Antibacterial Screening by Disc Diffusion Method:

Extract Used	**Diameter of Zone of inhibition (mm)**			
	Gram +ve Bacteria		**Gram -ve Bacteria**	
	B.subtilis	*S.aureus*	*E. coli*	*S.paratyphi*
Ciprofloxacin	31	29	26	13
MeOH.HMS	15	14	12	10
Control	8	7	9	8

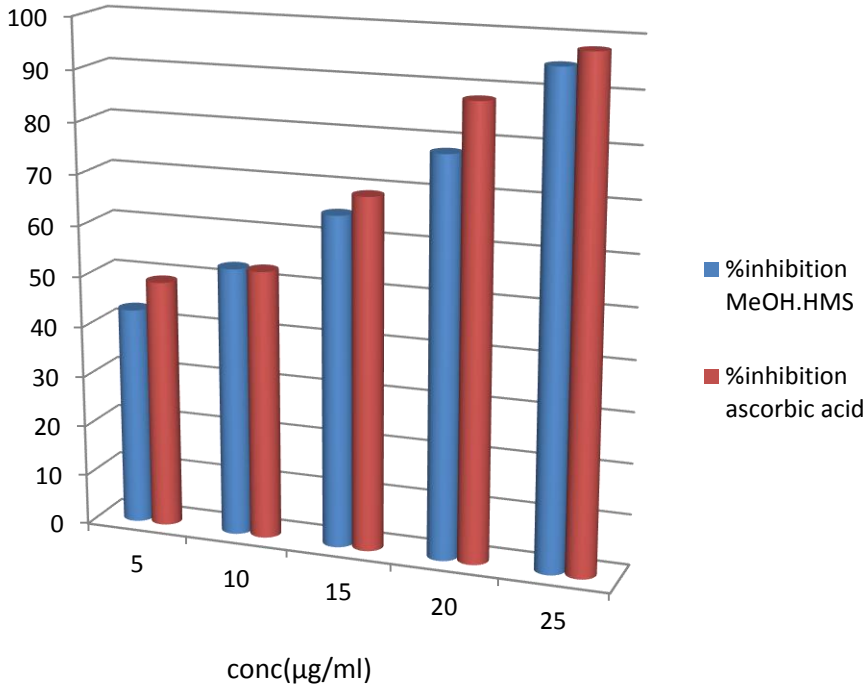
- Antifungal Screening by Disc Diffusion Method

Extract Used	**Diameter of Zone of Inhibition (mm)**	
	Candida albicans	***Aspergillus niger***
Clotrimazole	30	32
MeOH. HMS	15	16
Control	7	7

3. *INVITRO* ANTIOXIDANT STUDIES:

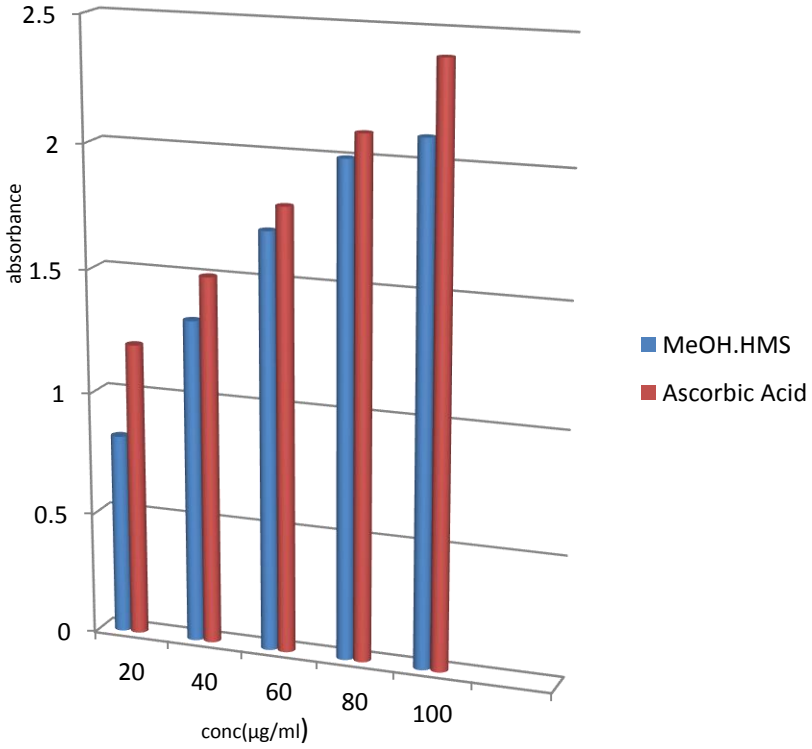
DPPH Assay

CONC(µg/ml)	% INHIBITION	
	MeOH. HMS	ASCORBIC ACID
5	43.22±0.15	49.76±0.16
10	53.07±0.17	53.08±0.17
15	65.77±0.16	69.47±0.18
20	78.33±0.15	88.42±0.14
25	95.33±0.13	98.75±0.13
EC 50	9.4±0.13	9.4±0.15



FRAP assay

CONC. (µg/ml)	ABSORBANCE	
	MeOH.HMS	ASCORBIC ACID
20	0.8287	1.2788
40	1.3264	1.5477
60	1.7279	1.8804
80	2.0407	2.1987
100	2.195	2.4273



4. EVALUATION OF OINTMENT:

- Physiochemical Evaluation

Parameters	Observation
Colour	Dark green
Odour	Characteristic
pH	5.8
Viscosity	16.05cps
Melting point	38°C
Spreadability(seconds)	12
Extrudability	14.2g

- HPTLC Analysis:

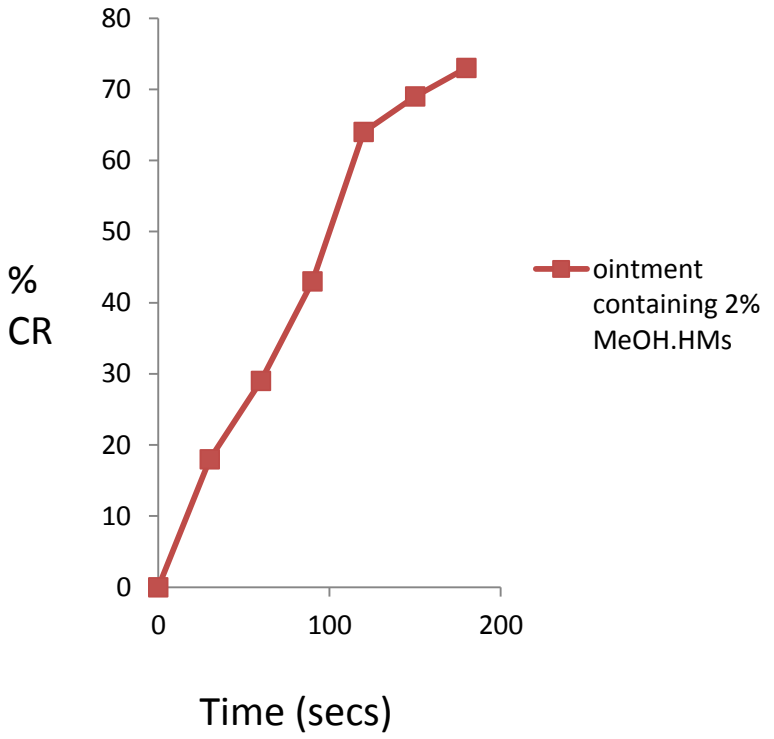
Sl.No	Samples	Maximum Rf	% area
1	HMME	0.84	7.81
2	Ointment containing 2% MeOH.HMS	0.83	9.85
3	Quercetin	0.83	87.89

HMME- *Hypericum mysorense* methanolic extract

From the above result, it can be concluded that the methanolic extract of *Hypericum mysorense* and the prepared ointment formulation contain quercetin as active ingredient.

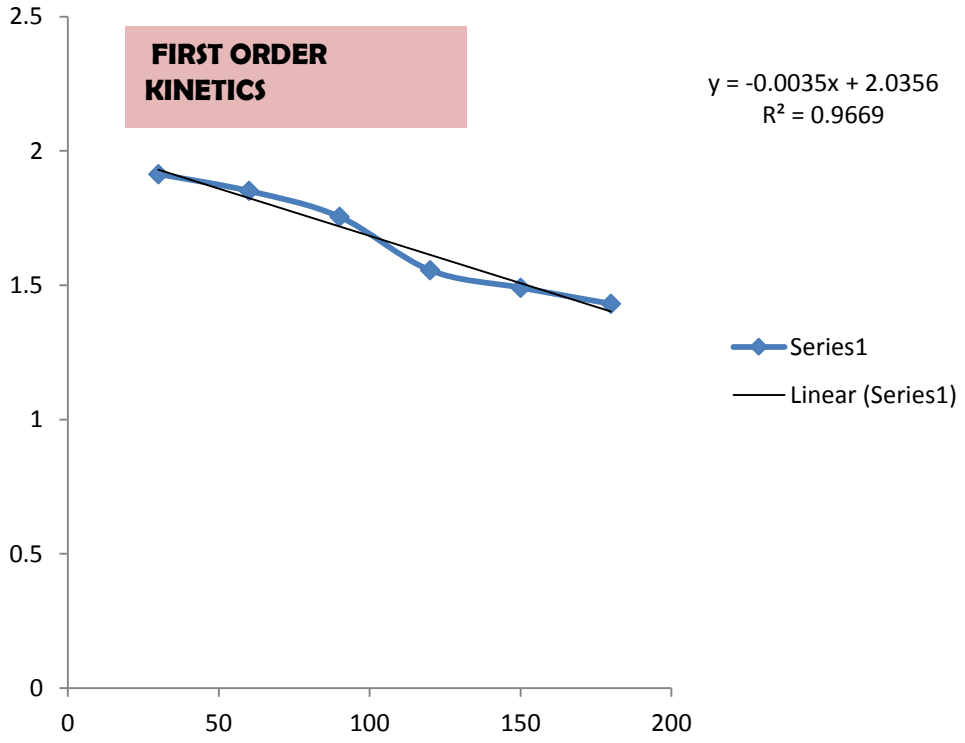
- *In-vitro* Diffusion Studies:

Time (secs)	%drug release
	Ointment containing 2% MeOH HMS
30	18
60	29
90	43
120	64
150	69
180	73



- Drug Release Kinetic Analysis :

Formulation	Zero order	First order	Higuchi model	
	R^2	R^2	R^2	n
2% Ointment containing methanolic extract	0.925	0.966	0.939	0.837



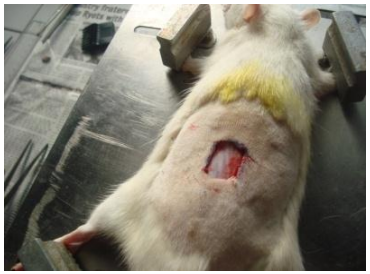
The data suggest that the formulation fit into first order equation for the release of drug that depends mostly on diffusion characterstics

•Stability Studies:

Sl.No	Formulation	Temp	Parameters					
			Colour	viscosity	Spreadabiliy	extrudability	MPt	Assay
1	Oint cointaining 2% extract (1st month)	4°	Dark green	20.15 cps	8	5.7g	38°	99.8%
		25°	Dark green	16.05 cps	10	13.8g	38°	
		40°	Dark green	16.01 cps	12	14.2g	38°	
2	Oint cointaining 2% extract (2nd month)	4°	Dark green	20.32 cps	7.5	5.5g	38°	98.9%
		25°	Dark green	15.98 cps	No change	No change	38°	
		40°	Dark green	15.95 cps	No change	No change	38°	
3	Oint cointaining 2% extract (3rd month)	4°	Dark green	20.3cps	7.4	5.3g	38°	98.5%
		25°	Dark green	No change	No change	No change	38°	
		40°	green	No change	No change	No change	38°	

Figure showing the wound healing activity from 0thday — 21th day.

EXCISION WOUND ON 0TH DAY



ONLY WOUND

WOUND + OINTMENT BASE

WOUND + OINT CONTAINING 2% MEOH.HMS

WOUND + STANDARD

EXCISION WOUND ON 3RD DAY

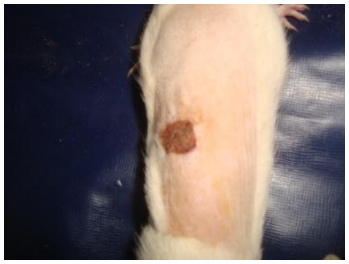
ONLY WOUND

WOUND + OINTMENT BASE

WOUND + OINT CONTAINING 2% MEOH.HMS

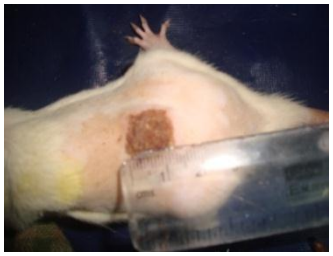
WOUND + STANDARD

EXCISION WOUND ON 5TH DAY



ONLY WOUND

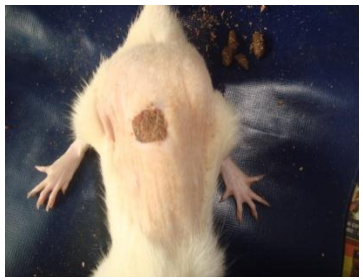
WOUND + OINTMENT BASE



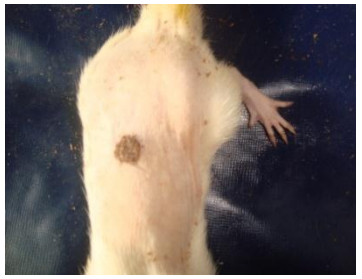
WOUND + OINT CONTAINING 2% MEOH.HMS

WOUND + STANDARD

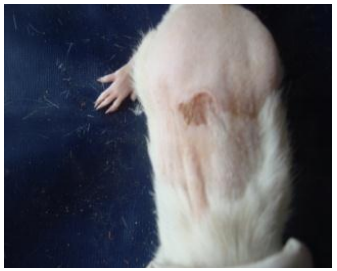
EXCISION WOUND ON 7TH DAY



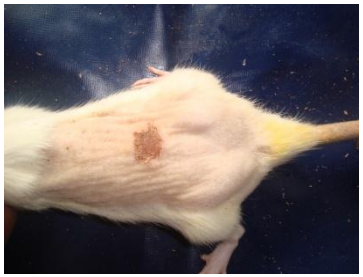
ONLY WOUND



WOUND + OINTMENT BASE



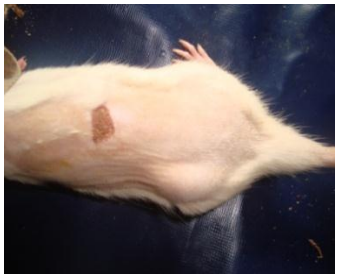
WOUND+ OINT CONTAINING 2% MEOH.HMS



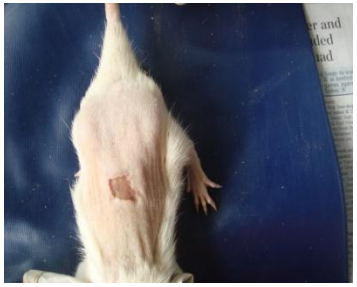
WOUND+ STANDARD

EXCISION WOUND ON 9TH DAY

ONLY WOUND



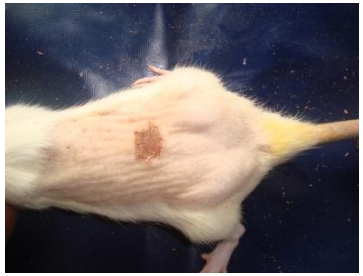
WOUND+OINTMENT BASE



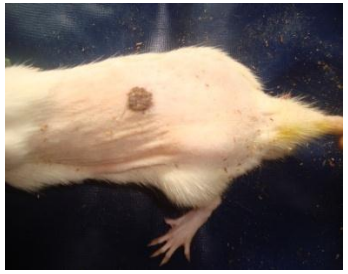
WOUND + OINT CONTAINING 2% MEOH.HMS

WOUND + STANDARD

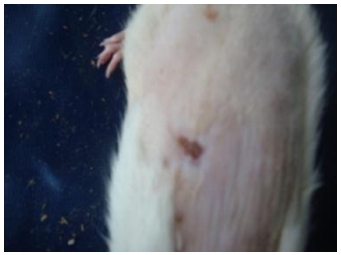
EXCISION WOUND ON 13TH DAY



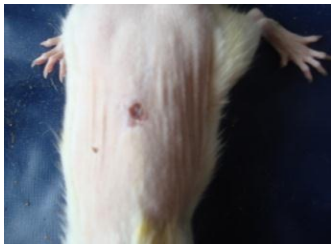
ONLY WOUND



WOUND+ OINTMENT BASE

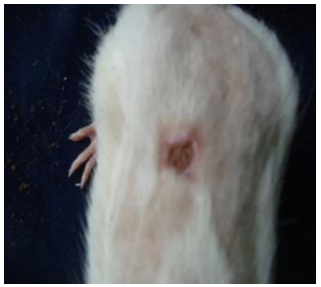


WOUND + OINT CONTAINING 2% MEOH.HMS



WOUND+ STANDARD

EXCISION WOUND ON 17TH DAY



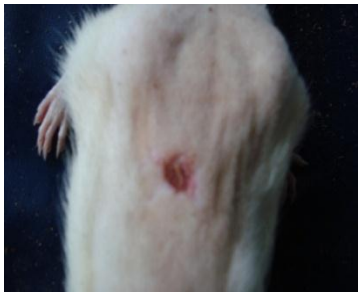
ONLY WOUND

WOUND+ OINTMENT BASE

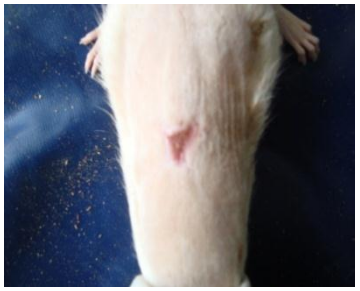
WOUND + OINT CONTAINING 2% MEOH.HMS

WOUND + STANDARD

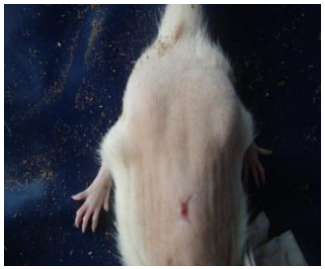
EXCISION WOUND ON 19TH DAY



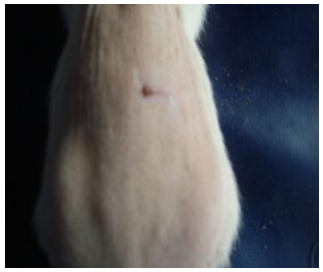
ONLY WOUND



WOUND + OINTMENT BASE



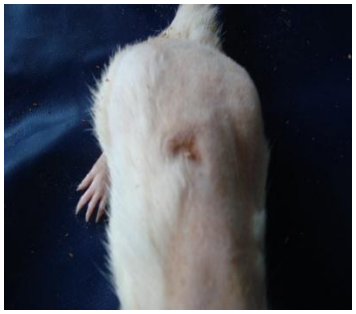
WOUND + OINT CONTAINING 2% MEOH.HMS



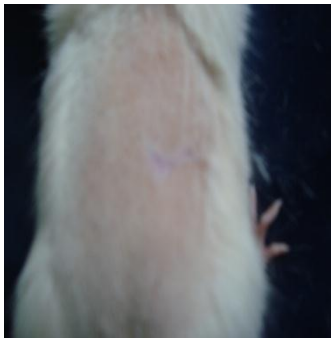
WOUND+ STANDARD

EXCISION WOUND ON 21ST DAY

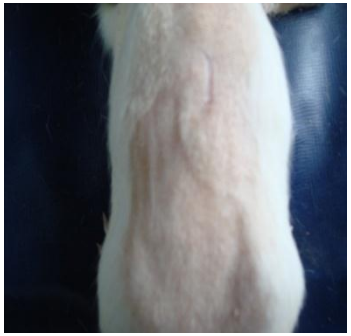
ONLY WOUND



WOUND + OINTMENT BASE



WOUND + OINT CONTAINING 2% MEOH.HMS



WOUND+ STANDARD

•Wound Healing Activity:

Percentage wound closure

Test Compounds	0th day	3rd day	7th day	13th day	17th day	19th day
	% WC	% WC	% WC	% WC	%WC	%WC
STD	0	15.7 ±0.29	39.8±0.77	81.8±1.09	88.5±1.25	95.5±1.10
OINTMENT BASE	0	13.5 ±0.49	27±0.83	52.5±1.24	66.8±1.24	88.2±1.88
CONTROL	0	27.5±0.58	23.6±2.03	74.2±0.99	72±0.99	90.1±0.99
OINTMENT CONTAINING 2%EXTRACT	0	10±0.42	13.8±1.48	55.4±1.08	84.1±1.08	96.9±1.08

Percentage Wound Reduction

Test compounds	0TH day	3rd day	7th day	13th day	17th day	19th day
	%WR	%WR	%WR	%WR	%WR	%WR
STD	100±0.00	84.3±0.29	60.2±0.77	18.2±1.09	11.5±1.25	4.42±1.10
OINTMENT BASE	100±0.00	86.5±0.49	73±0.83	47.5±1.24	33.2±1.24	11.8±1.88
CONTROL	100±0.00	72.5±0.58	76.4±2.03	25.8±0.99	28±0.99	9.81±0.99
OINTMENT CONTAINING 2% EXTRACT	100±0.00	90±0.42	86.1±1.48	44.5±1.08	15.8±1.08	3.02±1.08

Figure showing percentage wound closure

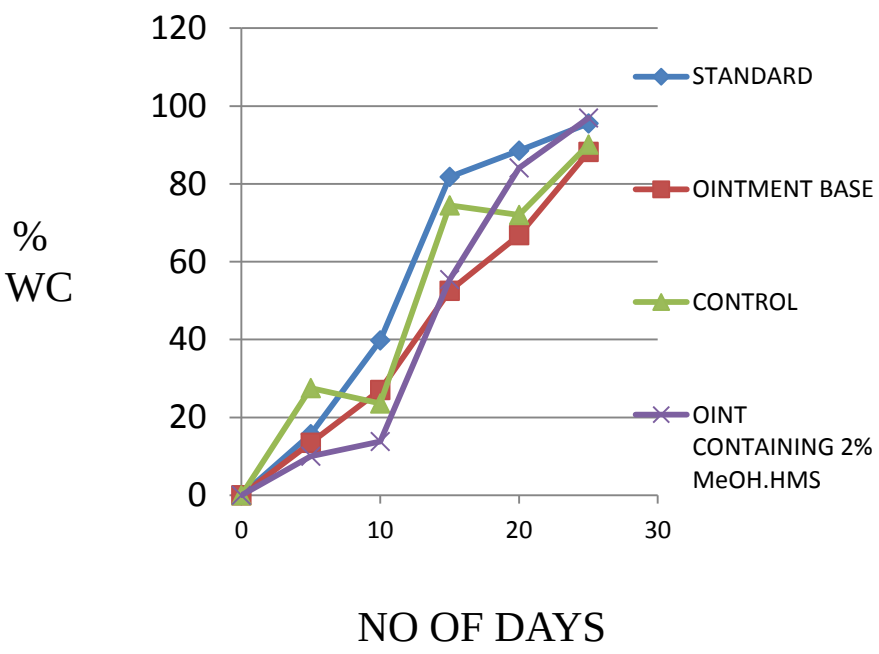
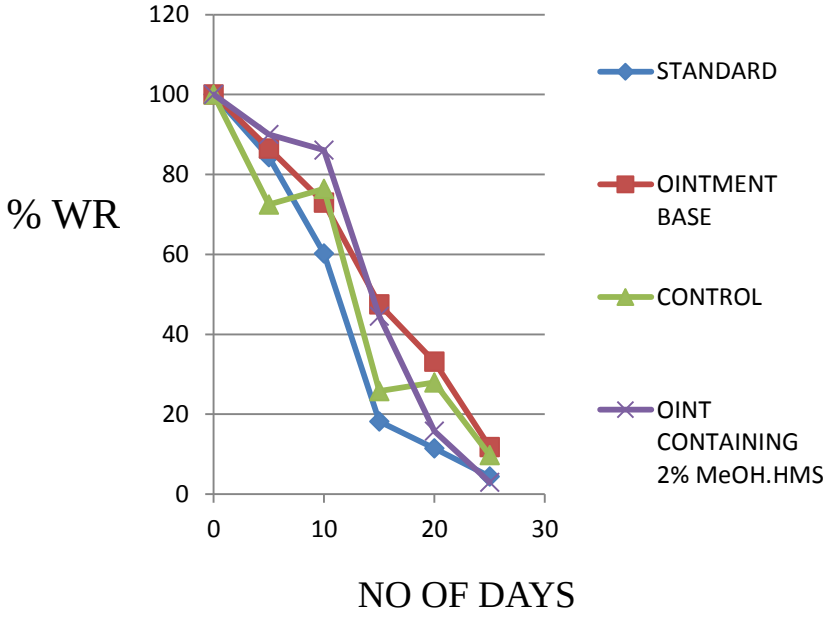
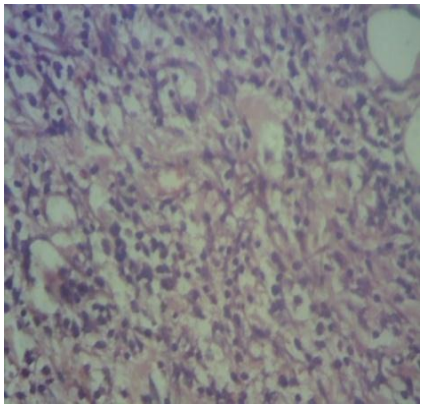


Figure showing percentage wound reduction

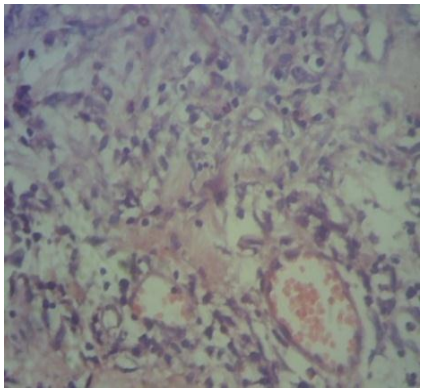


- Histopathological Studies:

  3rd day microscopical view of the wound tissue



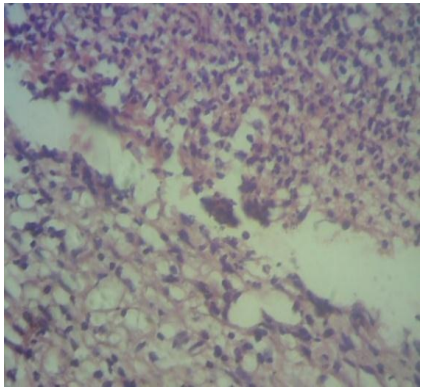
STANDARD



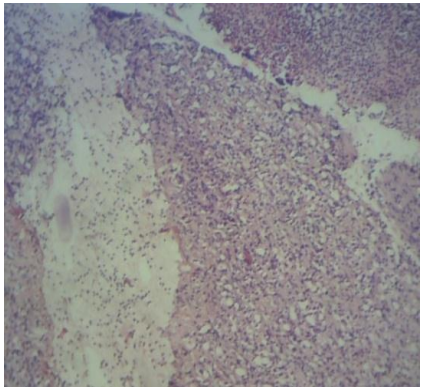
OINT CONTAINING 2% MeOH.HMS

Microscopic appearance: Section studied from the skin shows dense chronic inflammation composed of lymphocytes and plasma cells with early stage of granulation tissue. And mild fibrosis also noted.

9th day microscopical view of the wound tissue



STANDARD



OINT CONTAINING 2% MeOH.HMS

Microscopic appearance: Section studied from the skin shows granulation tissue with dense chronic inflammation along with scattered fibroblasts and proliferating papillae.

CONCLUSION

- Methanolic extract of *Hypericum mysorense* was prepared by continous hot percolation and was evaluated for its phytochemical property, *in-vitro* antimicrobial and *in-vitro* antioxidant activity. Using this methanolic extract, semi-solid dosage form (ointment) were formulated.
- Ointment were prepared with 2% methanolic extract using emulsifying ointment base. Both the formulations were then screened for its physiochemical property,HPTLCanalysis, *in-vitro* drug diffusion,kinetic studies and stability studies .
- The results of *in-vitro* antimicrobial and *in-vitro* antioxidant activity added on to the wound healing activity of the extract.

❑Hence, the 2% ointment was evaluated for its wound healing activity using excision wound model. The effect produced by the extract ointment, in terms of wound contracting ability, wound closure time and histopathological characteristics were comparable to that of a standard drug, povidone iodine ointment.

❑ Therefore, the present study concluded the wound healing activity of *Hypericum mysorense* at 2% concentration of the methanolic extract formulated as an ointment.

REFERENCES

1. Shilpashree, H. P. and Rai, Ravishankar (2009) *In vitro* plant regeneration and accumulation of flavonoids in *Hypericum mysorense*, International Journal of Integrative Biology, 8 (1, Cop). pp. 43-49. ISSN 0973-8363

2. Shanmugam Moorthi , *Hypericum mysorense:* A Potential Antioxidant and Antidepressant Folk Medicinal Plant of Nilgiris Biosphere-Western Ghats , ,Research Journal of Biotechnology Vol. 5(1) February (2010)

3. S. Gopinath, G. Sakthidevi, S. Muthukumaraswamy and V.R.Mohan, GC-MS Analysis of Bioactive Constituents of *Hypericum mysorense*, Journal of Current Chemical and Pharmaceutical Science: 3(1), 2013, 6-15 , ISSN 2277-2871

4. A *Osunwoke* Emeka, The Wound Healing Effect of Aqueous Leave Extracts of *Azadirachta Indica* on Wistar Rats, Journal of Natural Sciences Research ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online) Vol.3, No.6, 2013

THANK YOU

winCATS Planar Chromatography Manager

KMCH College of Pharmacy,
Kovai Estate, Kalapatty road,
Coimbatore-48

Analysis Report

SOP document		
Validated	Design	
Description :		
Analysis	D:\fractions\SANDEEP 28.1.14.cna	
Created/used by	KMCHCOP	Tuesday, January 28, 2014 7:06:42 PM
Current user	KMCHCOP	

Stationary phase

Executed by	KMCHCOP	Tuesday, January 28, 2014 5:30:04 PM
Plate size (X x Y)	10.0 x 10.0 cm	
Material	HPTLC plates silica gel 60 F 254	
Manufacturer	E. MERCK KGaA	
Batch		
GLP code		
Pre-washing	No	
Modification	No	

Definitions - Screening

Executed by	KMCHCOP	Tuesday, January 28, 2014 5:27:51 PM

Samples

HMME
2% OINTMENT
TGEAE
TGEE
TCEAE
TCEE
APIGENIN
RUTIN
QUERCETIN
TANNIC ACID

Substance name	Rf	Window size	Manufacturer	Batch number	Expiry date	Product number
RUTIN	0.00	1.000				
QUERCETIN	0.00	1.000				
APIGENIN	0.00	1.000				
TANNIC ACID	0.00	1.000				

Sample application - CAMAG Linomat 5

Instrument CAMAG Linomat 5 "Linomat5_170130" S/N 170130 (1.00.12)
Executed by KMCHCOP Tuesday, January 28, 2014 5:57:28 PM

Linomat 5 application parameters

Spray gas : Inert gas
Sample solvent type : Methanol
Dosage speed : 150 nl/s
Predosage volume : 0.2 ul

Sequence

Syringe size: 100 µl
Number of tracks: 10
Application position Y : 10.0 mm
Band length : 5.0 mm

No.	Appl. position	Appl. volume	Vial #	Sample ID	Active
>1	10.0 mm	2.0 µl	1	TGEAE	Yes
>2	18.8 mm	4.0 µl	2	APIGENIN	Yes
>3	27.6 mm	2.0 µl	3	TGEE	Yes
>4	36.4 mm	2.0 µl	4	HMME	Yes
>5	45.2 mm	2.0 µl	5	RUTIN	Yes
>6	54.0 mm	2.0 µl	6	QUERCETIN	Yes
>7	62.8 mm	2.0 µl	7	2% OINTMENT	Yes
>8	71.6 mm	2.0 µl	8	TANNIC ACID	Yes
>9	80.4 mm	2.0 µl	9	TCEAE	Yes
>10	89.2 mm	2.0 µl	10	TGEE	Yes

Development - Glass tank

Chamber type Twin Trough Chamber 10x10cm
Executed by KMCHCOP Tuesday, January 28, 2014 6:34:01 PM
Comment
Pre-conditioning
Mobile phase TOLUENE:ETHYL ACETATE:FORMIC ACID:METHANOL(3:6:1.6:0.4)
Solvent front position 70.0 mm
Volume 10.0 ml
Drying device Oven
Temperature 60 °C
Time 5 Minutes
Notes

Detection - CAMAG TLC Scanner 3

Information

Application position 10.0 mm
Solvent front position 70.0 mm

Instrument CAMAG TLC Scanner 3 "Scanner3_170310" S/N 170310 (1.14.28)
Executed by KMCHCOP Tuesday, January 28, 2014 7:01:43 PM
Number of tracks 10
Position of first track X 10.0 mm
Distance between tracks 8.8 mm
Scan start pos. Y 5.0 mm
Scan end pos. Y 75.0 mm
Slit dimensions 5.00 x 0.45 mm, Micro
Optimize optical system Light
Scanning speed: 20 mm/s
Data resolution: 100 µm/step

Measurement Table

Wavelength	254
Lamp	D2 & W
Measurement Type	Remission
Measurement Mode	Absorption
Optical filter	Second order
Detector mode	Automatic
PM high voltage	273 V

Detector properties

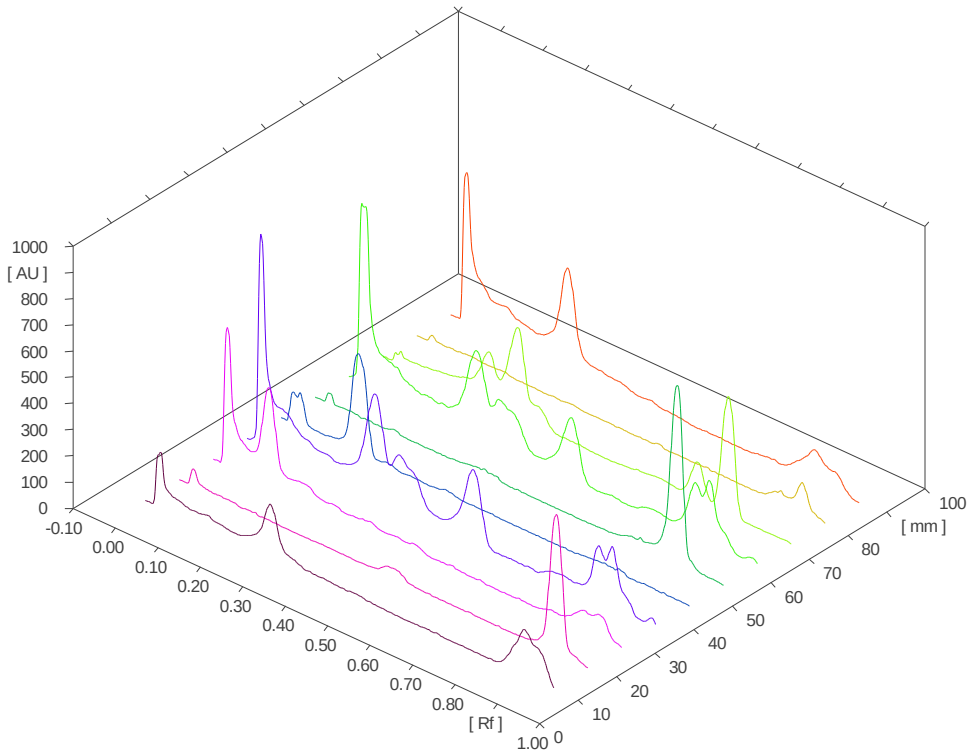
Y-position for 0 adjust	5.0 mm
Track # for 0 adjust	0
Analog Offset	10%
Sensitivity	Automatic (41)

Integration

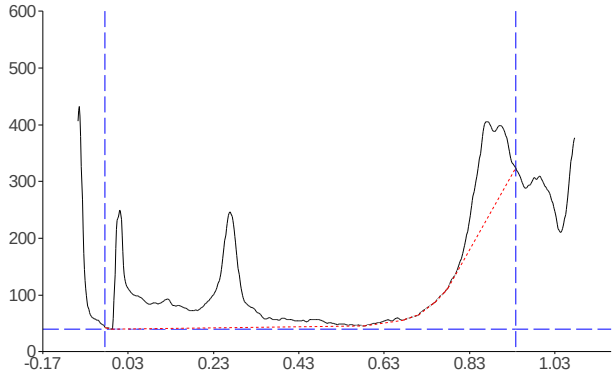
Properties

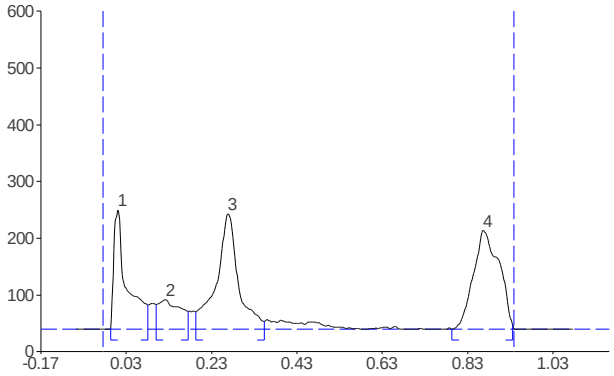
Data filtering	Savitsky-Golay 7
Baseline correction	Lowest Slope
Peak threshold min. slope	5
Peak threshold min. height	10 AU
Peak threshold min. area	50
Peak threshold max. height	990 AU
Track start position	8.9 mm
Track end position	66.8 mm
Display scaling	Automatic

All tracks at Wavelength



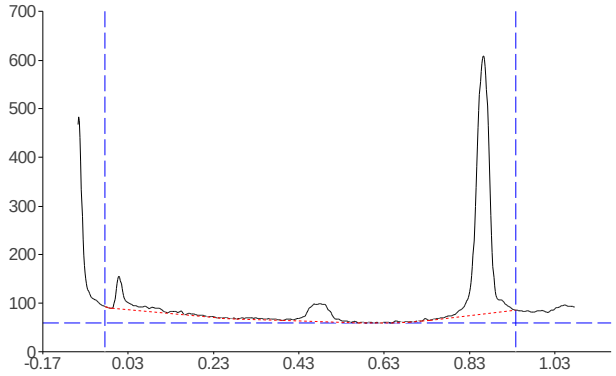
Track 1, ID: TGEAE

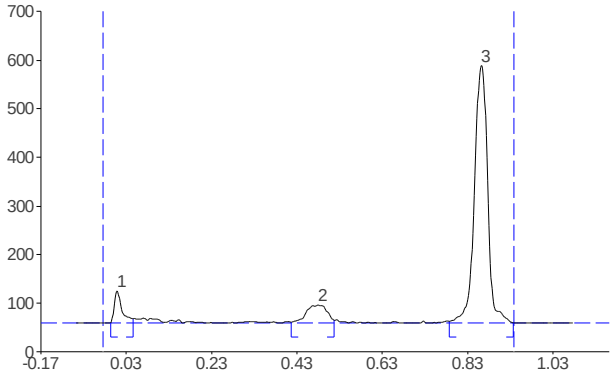




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	2.9	0.01	210.3	32.79	0.09	43.2	4535.2	21.50	unknown *
2	0.11	44.0	0.13	52.3	8.16	0.18	31.6	1943.8	9.21	unknown *
3	0.20	31.5	0.27	204.1	31.83	0.36	14.5	7250.7	34.37	unknown *
4	0.80	0.3	0.87	174.5	27.22	0.94	8.4	7364.6	34.91	unknown *

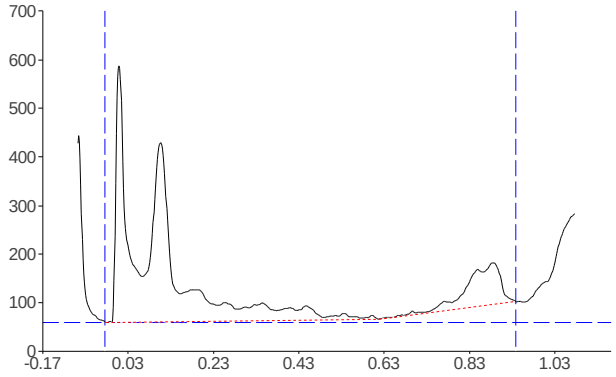
Track 2, ID: APIGENIN

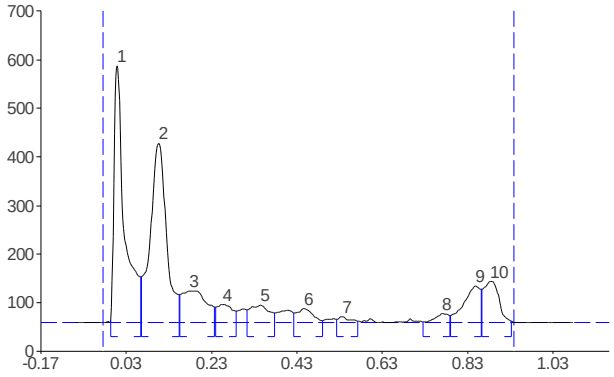




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	0.8	0.01	66.6	10.50	0.05	9.1	852.7	6.22	unknown *
2	0.42	2.3	0.48	37.6	5.92	0.52	6.3	1300.1	9.49	unknown *
3	0.79	3.8	0.87	530.2	83.58	0.94	0.3	11549.3	84.29	unknown *

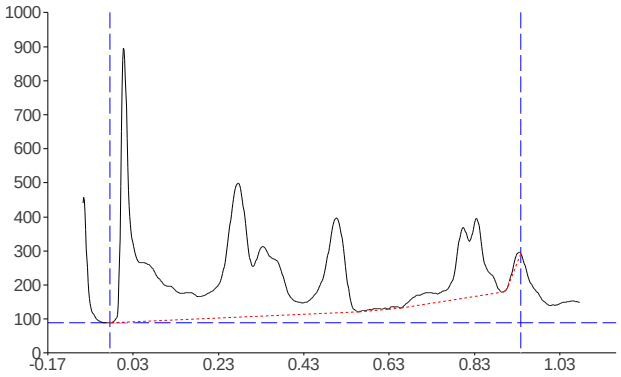
Track 3, ID: TGEE

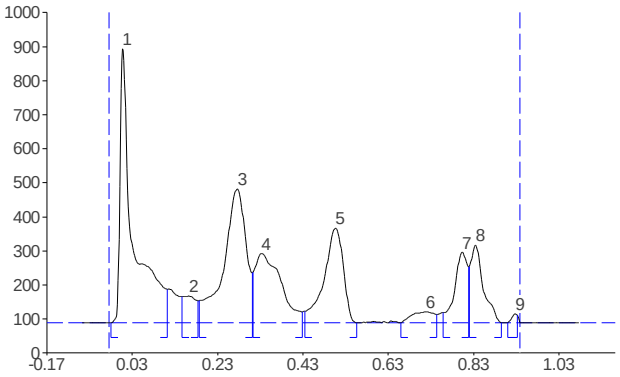




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	6.1	0.01	528.3	41.90	0.07	94.5	9041.7	30.89	unknown *
2	0.07	95.1	0.11	368.5	29.23	0.16	57.8	9742.4	33.28	unknown *
3	0.16	58.1	0.18	65.8	5.22	0.24	32.6	2717.2	9.28	unknown *
4	0.25	32.7	0.26	38.1	3.02	0.29	24.2	974.2	3.33	unknown *
5	0.32	27.0	0.35	36.6	2.90	0.38	20.3	1166.5	3.99	unknown *
6	0.43	20.3	0.45	29.6	2.35	0.50	4.8	774.4	2.65	unknown *
7	0.53	6.8	0.54	13.1	1.04	0.58	3.2	235.0	0.80	unknown *
8	0.73	1.7	0.78	19.7	1.56	0.80	14.4	421.5	1.44	unknown *
9	0.80	14.6	0.85	75.6	6.00	0.87	68.7	2056.0	7.02	unknown *
10	0.87	68.9	0.89	85.5	6.78	0.94	3.8	2141.2	7.32	unknown *

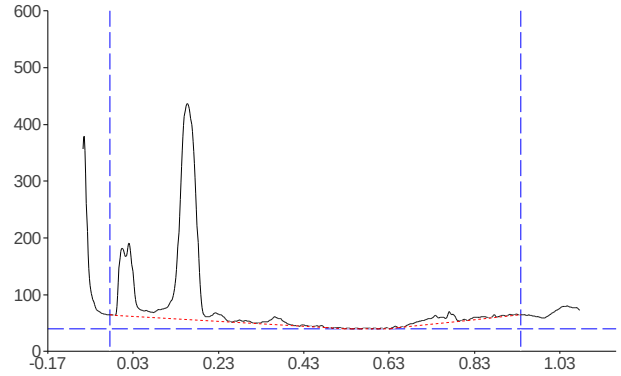
Track 4, ID: HMME

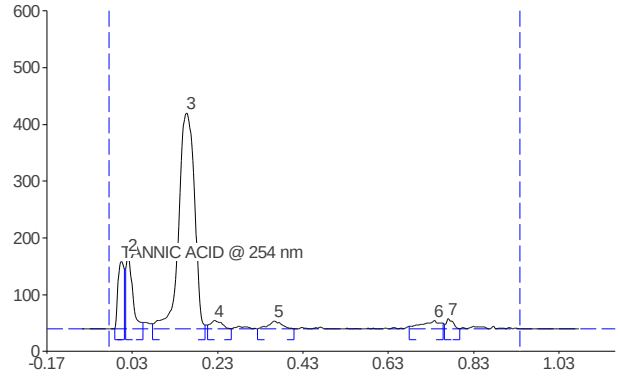




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	0.3	0.01	805.7	35.68	0.12	99.5	17296.7	28.08	unknown *
2	0.15	77.6	0.17	79.1	3.50	0.19	65.7	1727.6	2.80	unknown *
3	0.19	65.9	0.28	393.5	17.43	0.32	147.4	14489.1	23.52	unknown *
4	0.32	149.3	0.34	204.0	9.03	0.43	33.5	8627.6	14.01	unknown *
5	0.44	34.8	0.51	278.2	12.32	0.56	0.2	8794.8	14.28	unknown *
6	0.67	0.3	0.72	33.5	1.48	0.75	24.7	1246.1	2.02	unknown *
7	0.77	30.2	0.81	208.5	9.23	0.82	164.9	4375.5	7.10	unknown *
8	0.83	166.9	0.84	228.9	10.13	0.90	0.4	4810.5	7.81	unknown *
9	0.92	0.5	0.94	26.8	1.19	0.94	23.3	227.4	0.37	unknown *

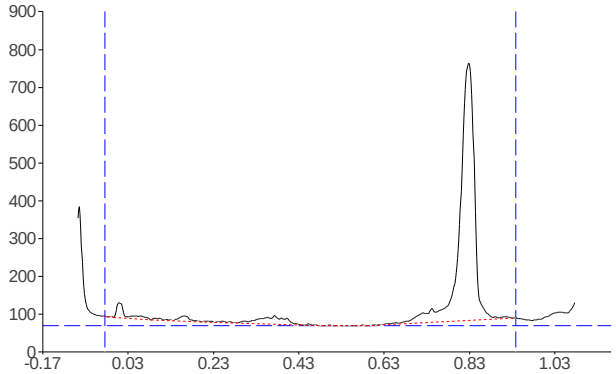
Track 5, ID: RUTIN

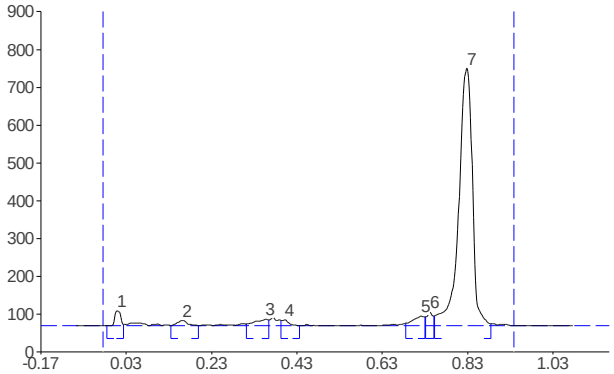




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	0.5	0.01	119.1	17.17	0.02	105.6	1217.9	8.84	TANNIC ACID
2	0.02	106.7	0.03	129.7	18.70	0.06	11.4	1483.9	10.77	unknown *
3	0.08	9.4	0.16	381.1	54.94	0.20	6.8	9797.4	71.11	unknown *
4	0.21	7.2	0.23	15.4	2.22	0.27	0.3	290.4	2.11	unknown *
5	0.33	0.9	0.37	14.1	2.03	0.41	0.0	319.8	2.32	unknown *
6	0.69	4.6	0.74	15.2	2.20	0.77	10.2	453.4	3.29	unknown *
7	0.77	8.2	0.78	19.0	2.74	0.80	1.0	215.5	1.56	unknown *

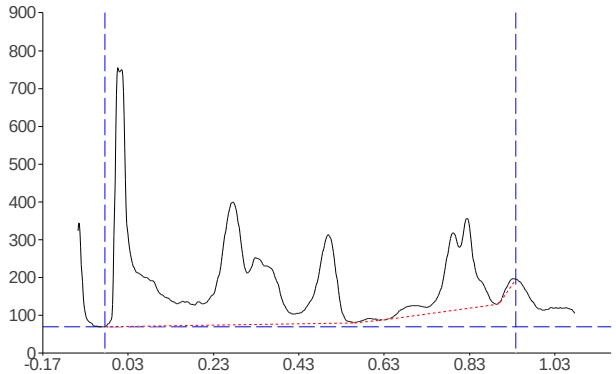
Track 6, ID: QUERCETIN

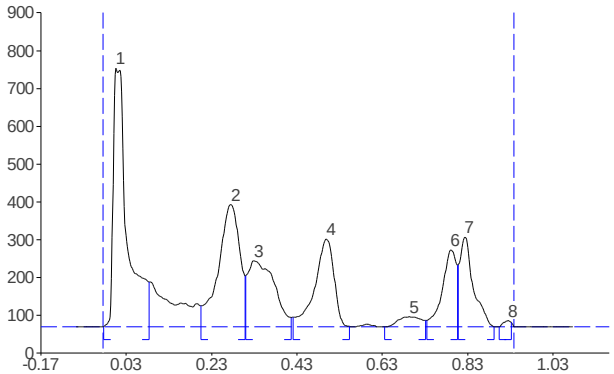




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.01	0.7	0.01	39.6	4.76	0.03	3.6	390.4	2.25	unknown *
2	0.14	1.2	0.17	14.6	1.76	0.20	1.4	256.0	1.47	unknown *
3	0.32	5.2	0.36	17.3	2.08	0.37	15.3	399.1	2.30	unknown *
4	0.40	15.2	0.41	16.8	2.02	0.44	0.5	200.7	1.16	unknown *
5	0.69	6.8	0.73	26.0	3.13	0.74	23.6	498.9	2.87	unknown *
6	0.74	23.7	0.75	36.4	4.38	0.76	26.1	357.2	2.06	unknown *
7	0.76	26.6	0.83	680.4	81.86	0.89	5.4	15257.9	87.89	unknown *

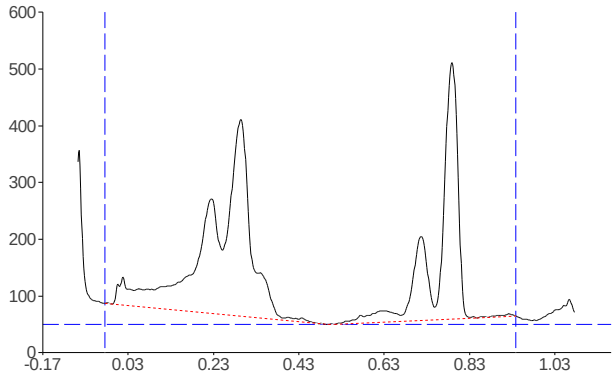
Track 7, ID: 2% OINTMENT

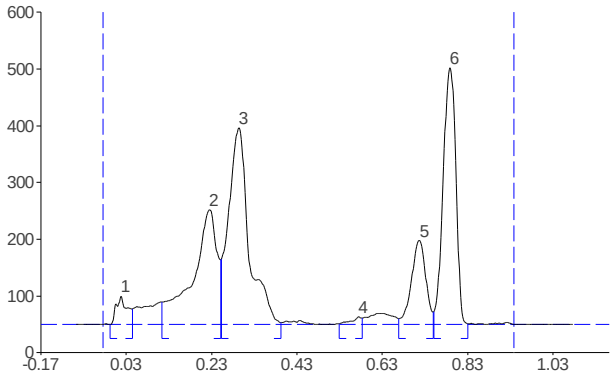




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.02	0.8	0.01	685.0	35.98	0.09	119.2	15821.6	29.62	unknown *
2	0.21	56.0	0.28	324.0	17.02	0.31	135.3	11339.5	21.23	unknown *
3	0.32	135.7	0.34	175.8	9.23	0.42	25.3	7657.4	14.33	unknown *
4	0.43	26.1	0.51	232.9	12.24	0.56	2.0	7314.5	13.69	unknown *
5	0.64	0.3	0.70	27.1	1.42	0.74	17.4	1050.6	1.97	unknown *
6	0.74	17.8	0.80	204.3	10.73	0.81	163.8	4772.7	8.93	unknown *
7	0.81	165.3	0.83	237.6	12.48	0.90	0.6	5264.1	9.85	unknown *
8	0.91	0.5	0.93	16.9	0.89	0.94	12.3	202.3	0.38	unknown *

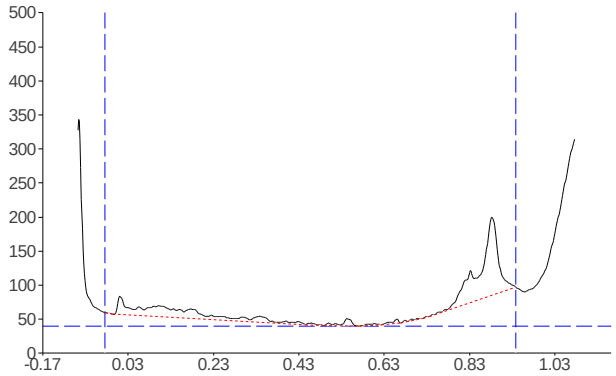
Track 8, ID: TANNIC ACID

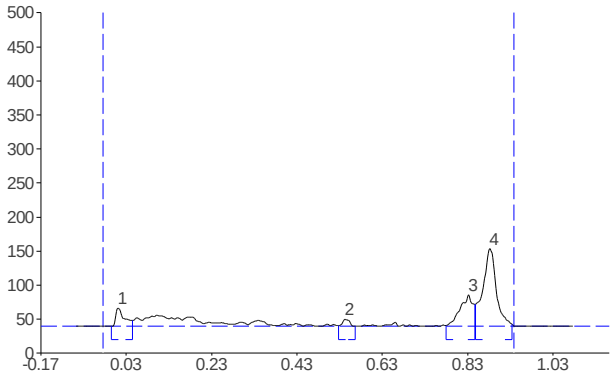




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	-0.00	0.3	0.02	49.9	4.11	0.05	28.2	914.9	2.71	unknown *
2	0.12	39.7	0.23	202.5	16.68	0.26	114.0	8024.8	23.80	unknown *
3	0.26	115.4	0.30	347.3	28.60	0.40	3.8	11607.0	34.43	unknown *
4	0.54	1.8	0.58	14.2	1.17	0.59	11.3	237.6	0.70	unknown *
5	0.68	10.9	0.72	147.9	12.18	0.76	22.3	3506.6	10.40	unknown *
6	0.76	23.3	0.80	452.6	37.27	0.84	1.6	9423.2	27.95	unknown *

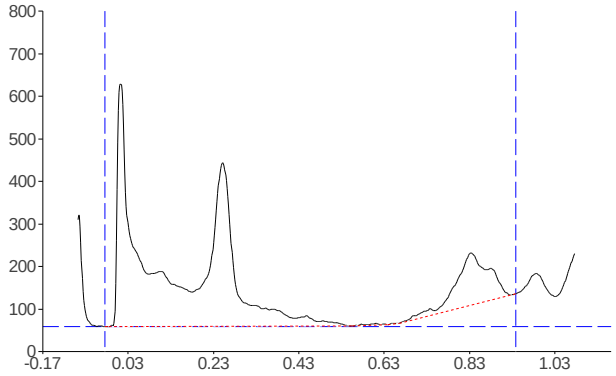
Track 9, ID: TCEAE

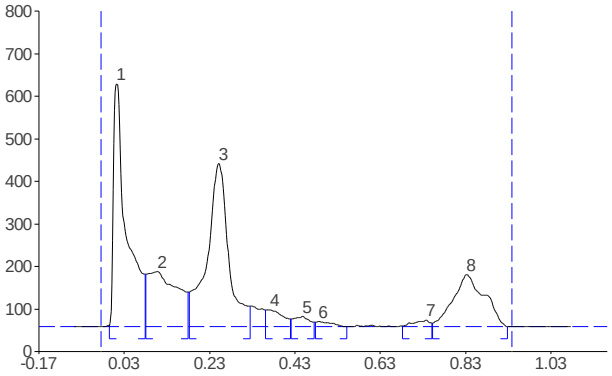




Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	0.0	0.01	27.1	13.65	0.05	8.8	377.7	9.48	unknown *
2	0.53	1.5	0.55	10.5	5.27	0.57	0.5	123.4	3.10	unknown *
3	0.79	1.1	0.84	46.8	23.55	0.85	32.6	984.2	24.71	unknown *
4	0.85	32.7	0.89	114.3	57.53	0.94	3.5	2498.2	62.71	unknown *

Track 10, ID: TGEE





Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.00	1.5	0.02	571.0	43.81	0.08	123.5	12246.2	29.89	unknown *
2	0.09	123.5	0.11	129.7	9.95	0.19	81.6	6499.1	15.86	unknown *
3	0.19	81.7	0.26	384.8	29.53	0.33	48.5	13471.9	32.88	unknown *
4	0.37	39.1	0.38	40.2	3.09	0.42	18.3	1110.5	2.71	unknown *
5	0.43	18.4	0.45	25.4	1.95	0.48	11.2	624.8	1.52	unknown *
6	0.48	10.5	0.49	12.6	0.97	0.56	0.1	326.9	0.80	unknown *
7	0.69	1.9	0.74	16.5	1.27	0.76	8.8	414.0	1.01	unknown *
8	0.76	8.9	0.84	123.0	9.44	0.94	0.3	6279.9	15.33	unknown *

Spectrum scan

Executed by	KMCHCOP	Tuesday, January 28, 2014 6:48:22 PM
Mode	All detected peaks	
Slit dimensions	5.00 x 0.45 mm, Micro	
Optimize optical system	Resolution	
Scanning speed	100 nm/s	
Data resolution	1 nm/step	
Reference spectrum, pos X	10.0 mm	
Reference spectrum, pos Y	10.0 mm	

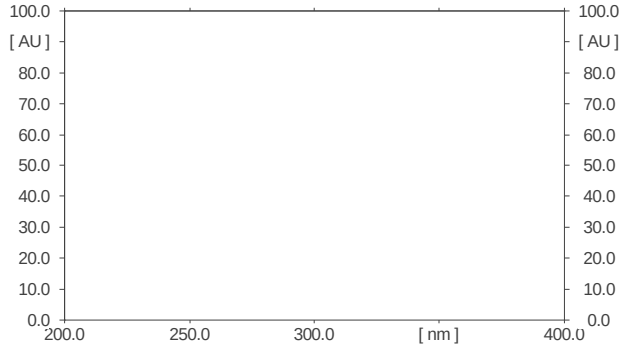
Measurement Table

Lamp	D2 & W
Start wavelength	200 nm
End wavelength	400 nm
Measurement type	Remission
Measurement Mode	Absorption
Optical filter	Second order
Detector Mode	Automatic

Detector properties

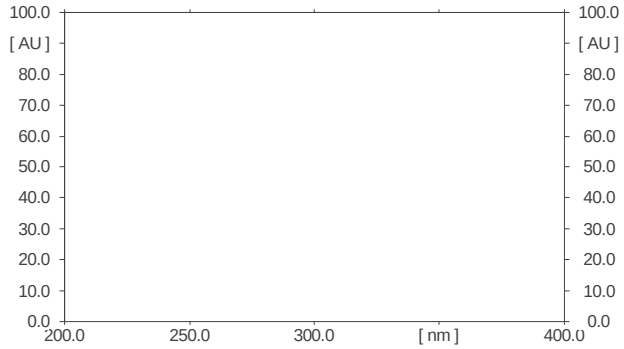
Y-position for 0 adjust	0.0 mm
Track # for 0 adjust	0
Analog Offset	10%
Sensitivity	Automatic (31)

RUTIN on all Tracks



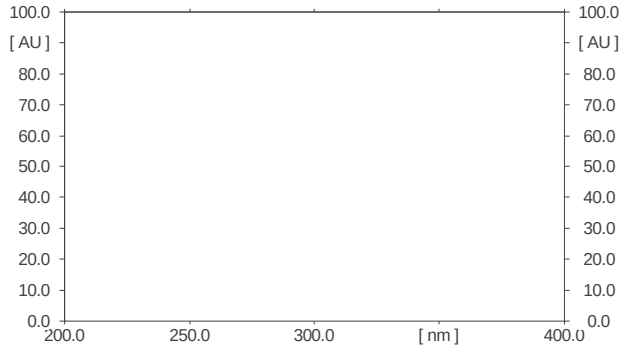
T	Rf	Substance	Max. @

QUERCETIN on all Tracks



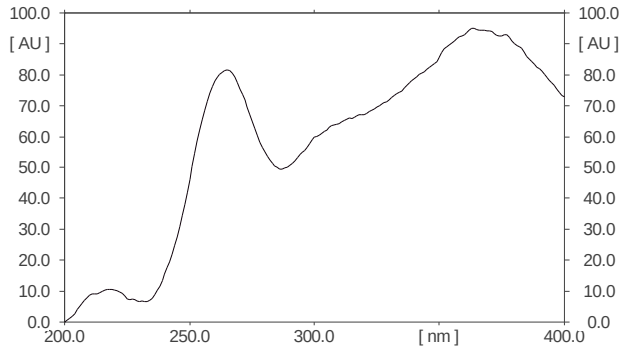
T	Rf	Substance	Max. @

APIGENIN on all Tracks



T	Rf	Substance	Max. @

TANNIC ACID on all Tracks



T	Rf	Substance	Max. @
5	0.01 Rf	TANNIC ACID	364 nm

Evaluation results

Evaluation Sequence

Track	Track type	Vial	Sample ID
1	Sample	1	TGEAE
2	Sample	2	APIGENIN
3	Sample	3	TGEE
4	Sample	4	HMME
5	Sample	5	RUTIN
6	Sample	6	QUERCETIN
7	Sample	7	2% OINTMENT
8	Sample	8	TANNIC ACID
9	Sample	9	TCEAE
10	Sample	10	TGEE

Table of substances

	Position	Tracks									1
Substance	MD mm	1	2	3	4	5	6	7	8	9	0
RUTIN	10.0										
QUERCETIN	10.0										
APIGENIN	10.0										
TANNIC ACID	10.0					A					

Documentation

Executed by	KMCHCOP	Tuesday, January 28, 2014 7:06:39 PM

Image Document

Executed by	KMCHCOP	Tuesday, January 28, 2014 7:05:00 PM
Image name	IMG_7208	

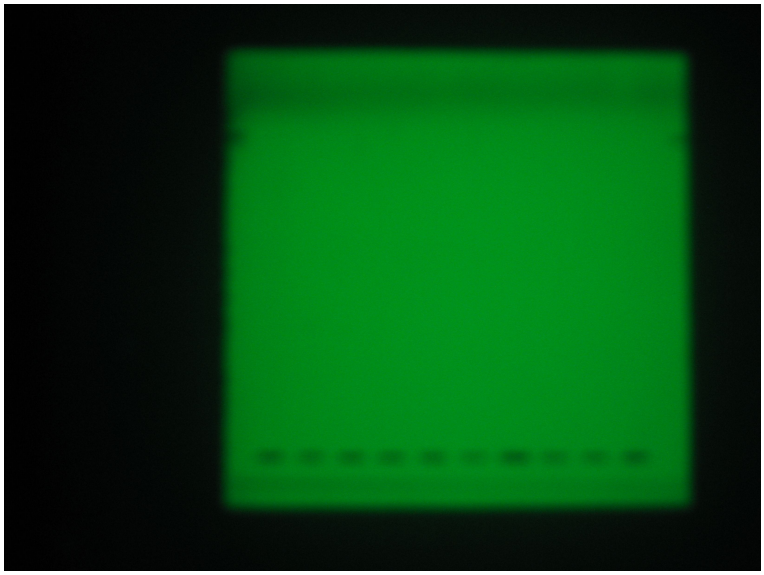


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Image ID	52E7B204

Image Document

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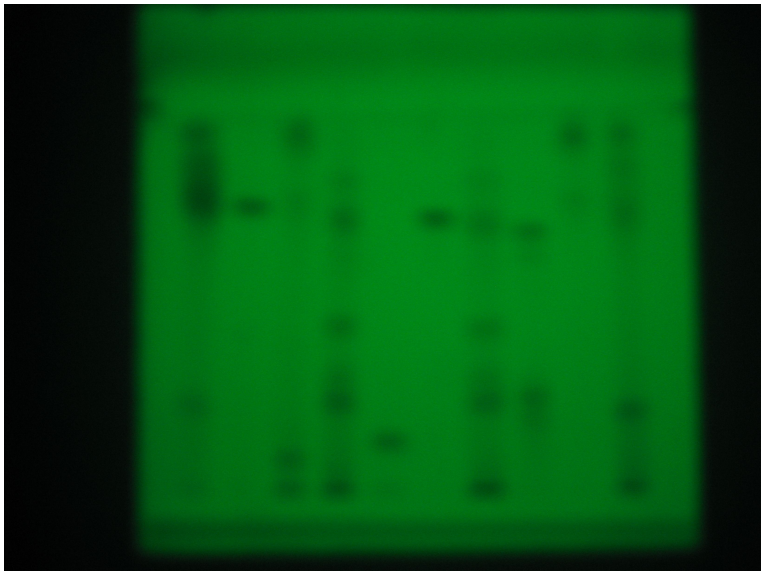
Image name	IMG_7209	

Created by	KMCHCOP on Tuesday, January 28, 2014 7:05:54 PM
Image ID	52E7B23A

Image Document

Executed by	KMCHCOP	Tuesday, January 28, 2014 7:06:37 PM
Image name	IMG_7211	



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